

# **Aspects of adiposity in ponies**

*Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy*

by

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# Abstract

## Aspects of adiposity in ponies. Alexandra H.A. Dugdale

Obesity is a growing problem for humans and their horses and ponies, yet emaciated animals still form an important part of the work of equine welfare charities. Non-invasive yet reliable methods of assessing equine body fat are required in order to promote management procedures to improve animal welfare. The overall objective of this work was to investigate the application of a horse-specific body condition scoring system in ponies in order to either validate or revise it, or even replace it with a novel system if necessary.

Seasonal differences in appetite, body mass (BM) gain, body condition score (BCS) change and direct (ultrasound) and indirect (morphometry and D<sub>2</sub>O dilution) measures of body fat were explored in two relatively homogeneous groups of mature Welsh mountain pony mares, studied over summer (June –September 2007) and winter (January-April 2008). The ponies in each group were paired so that, at study outset, two ponies were ‘thin’ (BCS, 1-3/9); two were ‘moderate’ (BCS, 4-6/9); and two were ‘obese’ (BCS, 7-9/9). The greatest appetites (peak 4.6% BM as DMI), increases in body mass (~60kg) and in BCS (~3 points) were recorded for ponies of non-obese outset condition in summer (non-ObS, n=4). For ponies of non-obese outset condition in winter (non-ObW, n=3), appetites peaked at 3.5% BM as DMI, BM increased by a mean of 50kg and BCS increased by ~2 points over the 3 month study period. Appetites for all obese (Ob, n=4) ponies remained almost constant (~2% BM as DMI; peak 2.3% BM as DMI) and minimal changes in BM (n=3) and BCS (n=4) were recorded, regardless of season. All measures of body fat increased for non-Ob ponies (non-ObS>non-ObW). An exponential relationship was determined between body fat content and BCS and for values > 6, BCS was not a useful predictor of actual body fat content. The endogenous circannual mechanisms to encourage winter weight loss were insufficient to prevent the development of obesity in *ad libitum* fed ponies.

The effects of dietary restriction to 1% BM as DMI were studied in a group of 5 overweight or obese mature pony mares (BCS 5.6-8/9). Those measures outlined above were likewise recorded. All ponies remained healthy throughout the 12 week trial. Overall, BM reduced by 1% of outset BM per week. Approximately half the lost BM comprised fat, but fatter animals lost relatively more fat. Despite an average loss of ~30 kg BM, BCS did not change appreciably suggesting that BCS was a relatively poor indicator of early weight/fat loss in obese ponies.

The relationships between BCS, direct (ultrasonic) and indirect (morphometric and D<sub>2</sub>O dilution derived) measures of body fat and actual body fat content determined by both physical dissection and chemical cadaver analysis were explored using 7 donated mature Welsh pony mares (BCS 1.25 to 7/9). Body ‘fat’ content (dissected white adipose tissue or chemically-extracted lipid fractions) was the most variable constituent of the cadavers (up to 1/3<sup>rd</sup> body mass), and was non-linearly related to BCS. From these studies, it was also possible to validate the D<sub>2</sub>O dilution technique for the measurement of total body water and fat in ponies.

Contemporaneously gathered data for BCS and body fat (D<sub>2</sub>O dilution) from 48 separate observations were explored statistically. A non-linear association between body fat content and BCS was confirmed, with a cut off value of BCS 7/9, above which BCS was less useful for determining body fat content. A novel BCS system was created and is undergoing field trials.

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# **Chapter 1**

## **General Introduction**

## 1.1 The British National herd and Native ponies

The ancestors of modern horses and ponies inhabited highly seasonal environments. The origins of the horse extend back to the Eocene age, around 50-30 million years ago, after the Palaeocene which followed the massive extinction of the dinosaurs at the end of the Cretaceous period. In the Eocene age, animals like Hyracotherium (popular name: Eohippus), a fox-sized, forest-dwelling mammal and the later Meshippus, flourished. The following Miocene (5-20 million years ago) and Pliocene (2.5-5 million years ago) ages saw the development of animals such as the Merychippus, Hipparion and the Pliohippus, which had three toes on each foot. Finally, the Pleistocene age (the last 2-2.5 million years) saw the development of what we refer to as the modern 'horse', *Equus caballus*\*.

In Britain, the National herd currently comprises more than 1.3 million horses and ponies (National Equine Database). Within the National herd the exact proportion of ponies is unknown but is considered in excess of 350,000 (NED Administration Team, December 2010). Several pony breeds are considered Native to Britain and, in particular, the Exmoor pony has been considered a close ancestor of the first truly wild horses present here (Speed and Etherington, 1952). Such Native ponies were considered to 'show little or no signs of cross-breeding and selection by man, never be housed, fend for themselves and find all their own food' (Speed and Etherington, 1952). With domestication come the pit-falls of modern husbandry techniques and selective breeding which, according to Speed and Etherington (1951), will cause the demise of the domesticated animal. In the present crisis of growing obesity among the human and companion animal populations, it is interesting to ponder the place of modern management in the obesity epidemic in *Equidae*.

## 1.2 Obesity – an International epidemic

During the past two decades, public health concerns have increasingly been focused on the global epidemic of obesity plaguing particularly Western civilisations (Kopelman, 2000; Cummings and Schwartz, 2003). Obesity has been defined as the excessive accumulation of body fat which adversely affects health (NIH, 1985). However, not all fat depots are similar such that, at least for people, accumulation of upper body fat (subcutaneous and visceral) is more related to adverse metabolic consequences than lower body fat (Jensen, 2008). For humans, the consequences of obesity include features of the metabolic syndrome (cardiovascular disease, type 2 [insulin resistant] diabetes mellitus and renal disease), sleep apnoea/hypopnea and certain forms of cancer (DeFronzo and Ferrannini, 1991; Kopelman, 2000; Matthaei *et al.*, 2000; Reisin and Alpert, 2005; Bastard *et al.*, 2006).

More recently, veterinary surgeons have also noted an increase in the prevalence of obesity among companion animals which more or less parallels the magnitude of that seen in Western human populations, such that 40-50% of companion animals, be those animals dogs, cats, horses or ponies, are now considered to be overweight or obese (German, 2006; Thatcher *et al.*, 2007; Wyse *et al.*, 2008). The consequences of obesity in animals include insulin resistance, type 2 diabetes mellitus, orthopaedic disease, poor performance (athletic and reproductive) and, for *Equidae*, a predisposition to laminitis, which may be more a consequence of insulin resistance than obesity *per se* (Mattheeuws *et al.*, 1984; Nelson *et al.*, 1990; Kearns *et al.*, 2002; Kronfeld, 2004; Sillence *et al.*, 2006; Geor, 2008; Marshall *et al.*, 2009).

### 1.3 Pathophysiology of obesity-related disease

The increased white adipose tissue mass present in obese states is not merely an inert energy store or thermal insulator but is an active tissue secreting many adipocytokines (adipokines), the profile of which, in obese states, is such as to promote the development of insulin resistance, endothelial dysfunction and an inflammatory state (Trayhurn and Wood, 2004; Kim *et al.*, 2006; Vick *et al.*, 2007; Geor and Frank, 2009, Radin *et al.*, 2009). Hyperinsulinaemia was once heralded as a cardinal feature of insulin resistance (Draznin *et al.*, 2000), which was itself first defined by Kahn (1978) as “(existing) whenever normal concentrations of hormone (insulin) produce a less than normal biological response”. Hyperinsulinaemia only persists, however, for as long as insulin resistance is accompanied by a compensatory response (increased insulin secretion +/- decreased insulin clearance) in order to normalise or near-normalise blood glucose concentration (Kruszynska and Olefsky, 1996; Erdmann *et al.*, 2009; Toth *et al.*, 2010). When decompensation occurs, insulin secretion is reduced and marked hyperglycaemia follows, leading to type 2 diabetes mellitus in humans and small animals (Mattheeuws *et al.*, 1984; Nelson *et al.*, 1990; Bergman *et al.*, 2007). The equine pancreatic  $\beta$  cells are said to be less prone to exhaustion, so that type 2 diabetes mellitus is rare in horses (Durham *et al.*, 2008). Marked hyperinsulinaemia can develop in *Equidae*, the most severe consequence being endocrinopathic laminitis, a painful and debilitating inflammation of the digital lamellae which may ultimately require euthanasia (McGowan, 2008).

Insulin resistance is thought to affect mainly the metabolic actions (PI[3]K pathway) of insulin, possibly resulting in its mitogenic actions (MAPK pathway) being relatively over-stimulated by the increased availability of up-stream substrates

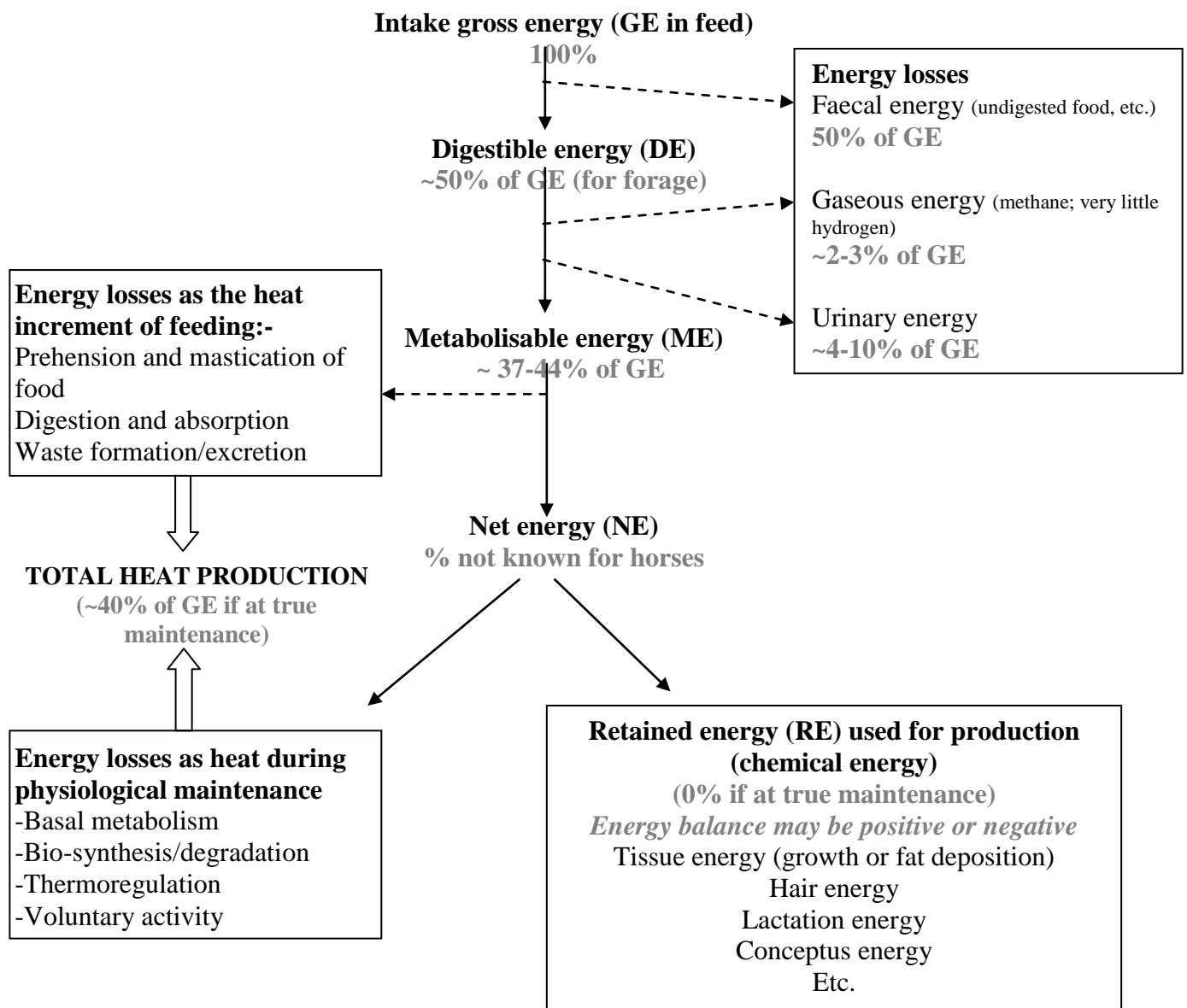
(Matthaei *et al.*, 2000; Kim *et al.*, 2006). From this model, the normal balance between insulin's effects to cause vasodilation (via nitric oxide (NO) as part of its metabolic functions) and vasoconstriction (via endothelins as part of its mitogenic functions) could be envisaged to favour vasoconstriction in insulin resistant states, this perhaps provoking equine laminitis (McGowan, 2008). However, the metabolic consequences of insulin resistance (in the liver, skeletal muscle and white adipose tissue) are also manifold, including relative hyperglycaemia and hypertriglyceridaemia with their own sequelae such as the production of advanced glyc(osyl)ation end-products (glucotoxicity) and advanced lip(oxid)ation end-products (lipotoxicity) which promote lipid peroxidation and cellular/mitochondrial dysfunction (Saltiel and Kahn, 2001; Unger and Zhou, 2001; Januszewski *et al.*, 2003; Boden and Laakso, 2004; Civitarese and Ravussin, 2008). In addition to obesity, other factors including physiological state (e.g. pregnancy), dietary composition and physical exercise affect insulin sensitivity (Freestone *et al.*, 1992; Storlien *et al.*, 2000; Tomas *et al.*, 2002; Hoffman *et al.*, 2003; Kronfeld *et al.*, 2005a and b). Emaciation (lipoatrophy) and lipodystrophy are also associated with insulin resistance, supporting an important role of adipose tissue itself (via the spectrum of adipokines it produces) in the regulation of insulin sensitivity (Saltiel and Kahn, 2001; Heilbronn *et al.*, 2004; Kronfeld *et al.*, 2005a).

#### **1.4 Origins of obesity: i) Regulation of food intake**

The reasons for the growing epidemic of human and companion animal obesity in modern societies are most likely manifold (including: genetics, environmental and social factors, little physical activity, *in-utero* nutrition, gastrointestinal microbiome) and remain the topics of much discussion (Kopelman, 2000;



Cummings and Schwartz, 2003; Cripps *et al.*, 2005; German, 2006; Gesta *et al.*, 2006; Sillence *et al.*, 2006; Speakman, 2006; Turnbaugh *et al.*, 2006; DiBaise *et al.*, 2008; Argo, 2009). Nevertheless, the final common pathway to obesity remains an excess of energy intake over energy requirement (Lowell and Spiegelman, 2003). The gross energy (GE) consumed in food is partitioned between the animal that consumes it and the various sources of energy loss as depicted for the horse in Figure 1.1.



**Figure 1.1:** The energy cascade (after Kienzle and Zeyner, 2010). For *Equidae*, gaseous losses and urinary energy losses are smaller than faecal losses (McDonald *et al.*, 2002; Fuller *et al.*, 2004; NRC, 2007).

## 1.5 Origins of obesity: ii) Quantifying energy requirements and intakes

The description of energy requirement for feeding purposes itself remains unresolved for animals (and man!), with no one system of energy evaluation being regarded as the ‘gold standard’ between all countries (Hill, 2006). For example, the energy requirements of horses for maintenance, growth, reproduction, lactation and exercise are expressed in terms of digestible energy (DE) in the UK, some parts of Europe, USA and Australasia, whereas in France, a net energy (NE) system is preferred (NRC, 2007). The metabolisable energy (ME) system is currently being revisited for horses in Germany (Kienzle and Zeyner, 2010). For ruminants, ME systems tend to be preferred while NE, DE or ME systems are variously used for pigs and poultry (McDonald *et al.*, 2002). While the NE system attempts to account for all sources of energy loss (for example, including the energy costs of feeding (prehension and mastication), digestion and absorption) and should therefore be more accurate, the system is more complex to use and the various sources of energy loss (and indeed, of energy ‘recovered’) do not always constitute a constant proportion of the metabolisable energy (ME) in the feed (NRC, 2007). To add to the confusion, the units of energy adopted by different countries also vary, the USA favouring calories, whereas Joules are preferred in the UK and elsewhere (1 calorie = 4.2 Joules).

Information regarding the energy requirements of *Equidae* for various physiological states (maintenance, growth, pregnancy, lactation and different levels of physical exercise) has been derived by various methods. These include: feeding trials; and indirect calorimetry (measurement of heat production estimated from the respiratory quotient: basal metabolic rate [fasting, resting, in thermoneutral environment], or maintenance [if nourished], or other [e.g. exercising]), which can be

performed either with well-fitting face masks (Burke and Albert, 1978; Pagan and Hintz, 1986b; Mazan *et al.*, 2003; Cruz *et al.*, 2006) or, more usually, when the subject is confined within a metabolic chamber (Burke & Albert, 1978; Kane *et al.*, 1979; NRC, 2007). The latter method requires correction factors to be applied for incomplete substrate oxidation, for example the production of methane (McDonald *et al.*, 2002). Alternative methods for quantification of energy requirements which can be used for free-ranging animals, involve either heart-rate monitoring or the administration of doubly-labelled water (DLW) (Butler *et al.*, 2004; Arnold *et al.*, 2006). Heart rate methods depend upon the Fick principle (relationship between cardiac output [heart rate x stroke volume] and oxygen uptake [difference between arterial and venous blood]), and assume that the oxygen consumption per heart beat ('oxygen pulse') is constant (or at least predictable), so enabling heart rate to be used to determine oxygen consumption (Butler *et al.*, 2004). The DLW technique, also requiring correction for methane production, compares the rates of turnover of  $^{18}\text{O}$  and  $^2\text{H}$  in plasma water which can provide indications of oxygen consumption and carbon dioxide production (Butler *et al.*, 2004; Fuller *et al.*, 2004).

Energy requirements may be expressed on a body weight or metabolic body weight ( $\text{BW}^{0.75}$  or even  $\text{BW}^{0.67}$ ) basis (NRC, 2007). Not surprisingly, the estimates differ according to the methodology employed, but provide a useful starting point (NRC, 2007). Further controversy, however, exists over the correct exponent which would most accurately scale for metabolic body mass (Pagan and Hintz, 1986a; NRC, 2007). Indeed, the subject of allometric scaling itself has become a complex battleground for mathematical modellers and biologists alike (West *et al.*, 1997; Brown *et al.*, 2002; Savage *et al.*, 2004).

If energy demand remains difficult to quantify accurately, what of energy intake? Chemical analyses of feedstuffs can provide accurate information regarding their composition from gross energy, through amino acid profiles and right down to mineral and vitamin contents (Anon, 2002). Feed intake can be measured, although this is more difficult for animals grazing pasture (NRC, 2007; Smith *et al.*, 2007; Ellis, 2010). Horses are trickle-feeders, spending 10-14hr/day grazing (Ellis, 2010). They naturally consume forage of high fibre content which undergoes extensive fermentation in the colon and caecum (McDonald *et al.*, 2002; Lawrence and Lawrence, 2009). Feeding trials enable estimation of the apparent digestibility of various dietary components including its gross energy content, thereby facilitating calculation of the digestible energy intake (Fuller *et al.*, 2001). In order to come full circle back to the consideration of energy requirements, extrapolation of digestible energy intake against average daily gain can then be used to estimate digestible energy requirements, for example, for maintenance (where ADG = 0 kg/day). Indirect calorimetry, however, offers more accurate estimations of energy requirements, despite the labour-intensive methodology (McDonald *et al.*, 2002).

### **1.6 Origins of obesity: iii) Satiety and adiposity**

The multifactorial nature of the control of food intake remains incompletely understood, although much attention has been directed to the study of the various food, environmental, behavioural, physiological and neuro-endocrine factors which affect it (Schwartz *et al.*, 2000; Illius *et al.*, 2002; Rhind *et al.*, 2002; Forbes, 2003; Zigman and Elmquist, 2003; Gossellin *et al.*, 2007; Woods and D'Alessio, 2008; Ellis, 2010). Studies in *ad libitum* fed ponies have reported that the animals failed to regulate their intakes according to requirements in the short-term (Cuddeford and

Hyslop, 1996; Argo *et al.*, 2002). Dulphy and colleagues (1997a & b) reported that the voluntary dry matter intakes of horses could not be predicted by the crude protein, crude fibre or neutral detergent fibre content of the feed. They also concluded that appetite was relatively poorly linked to season in horses compared to sheep, concluding that the organoleptic (sensory) characteristics of the food were more important short-term determinants of food intake in *Equidae* than sheep. Furthermore, since thorough chewing is a pre-requisite to small intestinal digestion and caeco-colic fermentation (McDonald *et al.*, 2002), Ellis (2010) hypothesised that such factors as taste preference and a requirement for chewing could over-ride short-term biochemical/neurohormonal regulators of food intake. The environmental and animal factors influencing short-term food intake have been outlined in Figure 1.2.

#### **Food/environmental factors**

- Availability (season; quantity; competition with other animals)
- Quality
- Variety
- Palatability (depends on physico-chemical and organoleptic properties)
- Availability of water (temperature/quality)
- Climate (local weather conditions; nuisance flies)



#### **Individual animal factors**

- Social status in herd hierarchy (may depend on breed, temperament, age, sex)
- Physiological status (seasonal changes dependent upon photoperiod)
- Health (e.g. dental disease)
- Prior experience of that food (neophobia)

**Figure 1.2:** Representation of some of the factors responsible for short-term regulation of food intake (after Vervuert and Bergero, 2010).

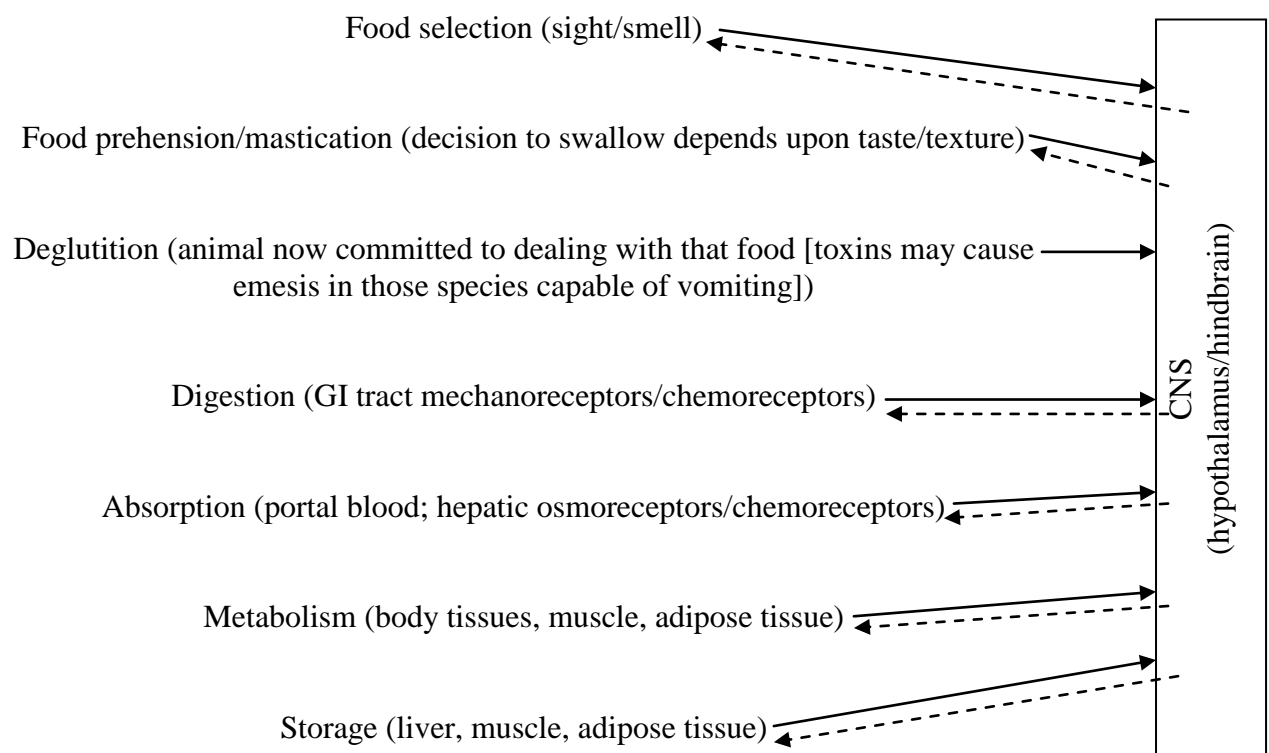
Physiological maintenance, growth and production require the support of sufficient energy and nutrient intake in both the short- and long-terms. Body mass, in particular body adiposity, is regulated in the long-term by a complex interplay of satiety/appetite signals and adiposity signals (Zigman and Elmquist, 2003; Redinger, 2008; Woods and D'Alessio, 2008).

Short-term satiety signals tend to be phasically active during meals and include mechanical (stretch receptors) and chemical signals (peptides e.g. ghrelin, cholecystokinin and PYY) deriving from the gastrointestinal tract. These satiety signals relay information to the central nervous system (particularly the arcuate nucleus, paraventricular nucleus, perifornical area and lateral hypothalamus and the dorsal vagal area of the brainstem) but also have local effects within the gastrointestinal tract to alter its motility and digestive function (Schwartz *et al.*, 2000; Cripps *et al.*, 2005; Gossellin *et al.*, 2007; Woods and D'Alessio, 2008).

Longer term adiposity signals, including insulin and leptin, are more tonically active and are thought to provide the central nervous system with an ongoing message proportional to the total body fat (energy store). These adiposity signals are capable of changing the sensitivity of the brain to satiety signals (Schwartz *et al.*, 2000; Woods and D'Alessio, 2008). Indeed, food intake has been described in terms of a satiety cascade involving both feed-back and feed-forward controls, with the central nervous system as the overall co-ordinator of food intake (Forbes, 2007; Figure 1.3 below).

To add to the complexity of the regulation of food intake, within the central nervous system the hypothalamic suprachiasmatic nucleus has been described as the

‘master-clock’ in its role as photoperiodically-entrained co-ordinator and regulator of circadian and seasonal physiological processes including metabolism (and food intake), sleep-wakefulness, hormone secretion (including those necessary for reproduction) and immune function (Van Cauter *et al.*, 1997; Thiery *et al.*, 2002; Henry, 2003; Murphy, 2010). These mechanisms may be of particular relevance to the long-term regulation of food intake in seasonal animals including *Equidae* (Arnold *et al.*, 2006).



**Figure 1.3:** Overview of the satiety cascade (after Forbes, 2007).

For mature animals, the long-term maintenance of a more or less stable body weight appears to be the end-point of energy homeostasis and the ultimate controller of food intake (Zigman and Elmquist, 2003; Woods and D'Alessio, 2008). In studies

of sheep, Vandermeersch-Doize and colleagues (1982 & 1983) reported a polyphasic response to *ad libitum* food provision wherein it took 6 months for body mass to stabilise, with body fat stabilising at around 37% of live weight.

For mature native animals of temperate latitudes, a seasonal oscillation of body mass around a more or less constant value occurs alongside other photoperiodically-entrained physiological adaptations (Suttie *et al.*, 1983; Mossberg and Jonsson, 1996; Argo *et al.*, 1999; Fuller *et al.*, 2001; Thiery *et al.*, 2002; Arnold *et al.*, 2006; Davies-Morel *et al.*, 2006). Fuller and colleagues (2001) confirmed that seasonal changes in appetite were photoperiodically-entrained in Native ponies, as changes were induced under artificial photoperiods. This seasonal effect has also been demonstrated for Przewalski horses studied in a semi-natural reserve, although mild winters and a relative abundance of good quality forage resulted in an overall increase in body weight over time in some animals with an attendant predisposition to laminitis (Budras *et al.*, 2001; Scheibe and Striech, 2001).

In short-term studies of increased or decreased food provision, alongside an overall increase or decrease in fatness, the order of fat (white adipose tissue) deposition at, or mobilisation from, different depots (fat patterning) has been studied in several species (Riney, 1955; Russel *et al.*, 1968; Westervelt *et al.*, 1976; Butler-Hogg, 1984; Butler-Hogg *et al.*, 1985; Gentry *et al.*, 2004). Although general patterns seem to emerge for some breeds and sexes, there remains a strong individuality regarding the preferred order of sites for fat deposition/mobilisation (Pond, 1998).



Some fat depots have a protective/structural role (e.g. periorbital fat, peri-articular fat) and some have a more metabolically active/functional role (e.g. pericardial fat, bone marrow fat and fat surrounding lymph nodes), and these depots tend to be the last to be mobilised in chronic starvation states (Pond, 1998). Other depots may be considered to have more of a storage function (e.g. retroperitoneal fat, subcutaneous fat) (Pond, 1998). That white adipose tissue from different depots can have different characteristics and be differentially involved in metabolic disease has been recognised for some time in man and remains an important focus for research in all species (Jensen, 2008; Burns *et al.*, 2010).

Body composition changes as an animal matures (Moulton, 1923; Callow 1948; Reid *et al.*, 1955; Lohman, 1971; Taylor *et al.*, 1989). Lean tissues such as bone and muscle are laid down preferentially as the animal grows, but white adipose tissue is the preferred form of chemical energy store once an animal has reached sexual maturity if energy intake exceeds demand, with repercussions for the meat industry (Reid *et al.*, 1955; Warriss, 2000; McDonald *et al.*, 2002; Sillence, 2004).

## **1.7 Body composition assessment**

Body composition, especially regarding body fat content, is important for animal health and productivity and also has physical, physiological, pharmacodynamic and pharmacokinetic implications which may affect an individual's response to pharmaceutical interventions (Clutton, 1988; Mansel and Clutton, 2008). The ability to assess and then, perhaps, manipulate the body composition of domestic species has wide ranging implications for the health and performance of production, competition and companion animals (Burkholder, 2000; Sillence, 2004). In particular,

with the increasing prevalence of obesity in both man and domestic animals, methods of assessing body fat content which are non-invasive, easy to apply in a clinical situation, reliable and cheap should promote animal welfare (German *et al.*, 2006).

Body composition can be described in terms of the relative amounts of the various biological or chemical components of the body. Traditionally, a two component model has been used, whereby the body is divided into fat and fat-free (lean) components and various assumptions are made regarding the nature of these two components (Wang *et al.*, 1992; Shen *et al.*, 2005). While the 2-component model remains useful for most animal studies, in which lean mass is said to be related to the patient's overall health (representative of overall protein nutrition), and the fat mass represents its energy reserves, each body can be divided into several components and body composition can be studied over 5 levels (atomic, molecular, cellular, tissue-system, whole-body) (Owens *et al.*, 1993; Wang *et al.*, 1992; Shen *et al.*, 2005; Schroder and Staufenbiel, 2006). No matter what method of assessment is used, however, body fat remains the most variable component of the body and the body's water and fat contents are inversely related (Siri, 1956; Lohman, 1971).

For humans, public health promotion has been an important aim of body composition research. One well known result is the body mass index (BMI; Quetelet's index), which, despite its shortfalls (no information about fat distribution; differences between sexes, ages and ethnicities) remains a useful screening test to forecast an increased risk of morbidity/mortality especially at the upper extreme of the scale (Sardinha and Teixeira, 2005). While BMI represents a globally recognised measure of body fatness in humans, albeit with some caveats depending upon the population to

which it is applied, no such similar measure currently exists for any animal species, although the subjective technique of body condition scoring (BCS) or various morphometric indices are often used as surrogate measures of the degree of fatness (Burkholder, 2000; Hayes and Shonkwiler, 2001). Although it is recognised that both emaciated and obese animals are less productive, quantification of the effects of varying body composition on health and productivity is necessary in order to define optimal body condition/s for different purposes (Jeffries, 1961; Andrew *et al.*, 1994).

The various methods available for studying body composition have differing advantages and disadvantages (Table 1.1). Many of these techniques, however, are not readily applicable to domestic species, especially larger species, due to the size and non-compliant nature of these animals.

Although non-destructive *in vivo* gold standard methods have been described for humans (densitometry and dual-energy X-ray absorptiometry), the gold standard, reference techniques for body composition studies in animals remain the destructive methods of chemical analyses and cadaver dissection (Jebb and Elia, 1993; Speakman, 2001) (Table 1.2). Unfortunately, comparison of destructive equine body composition studies is confounded by poorly recorded methods and lack of consistency in the techniques used, not only regarding the extent of the body (or its component/s) analysed but also in the exact chemical techniques used, particularly with reference to lipid extraction methodologies (Wang *et al.*, 1992) (Table 1.2). A similar lack of consistency is also found in the generally poorly validated techniques reported for evaluation of the various non-invasive methods of body composition assessment in *Equidae*, namely morphometric measurements of body shape and size

and ultrasonographic measurements of superficially accessible fat deposits (Hayes and Shonkwiler, 2001) (Table 1.3). Such non-invasive techniques, until validated against gold standard techniques, remain at best only rough guides. By comparison, several small animal techniques (Table 1.3) have been more rigorously validated (Laflamme 1997a & b; Burkholder and Thatcher, 1998; Speakman *et al.*, 2001). In the present ethical climate wherein destructive techniques should be replaced by non-destructive techniques where possible, there is a need for the development, refinement and thorough description of a non-destructive technique for the gold standard assessment of equine body composition against which other techniques can be validated.

**Table 1.1:** Comparison of the most commonly used techniques for the assessment of body composition. (The number of plus signs provides a relative rating index for accuracy and precision).

Technique	Accuracy	Precision	Advantages	Disadvantages
Chemical analysis	++++	Unknown	Accurate.	Cost (time, reagents, equipment). Destructive. Must define methodologies and how much of whole body is analysed.
Body weight	++	++++	Non-invasive. Easy to perform. Cheap. Often portable scales. Fast. No risk to patient. Can be used longitudinally to estimate changes in body composition in the individual.	Need calibrated weigh scales. No validated method to determine 'ideal' body mass. Unable to determine composition of weight changes.
Morphometry	+	+	Non-invasive. Easy to perform. Cheap. Fast. No risk to patient.	Prone to operator error. Best related to body composition only in the study population.
Skinfold thickness	+	+	Non-invasive. Relatively easy to perform, but operator training preferable. Fast. Cheap. Portable callipers. No risk to patient.	Prone to operator error. Not all patients (esp. Animals) easy to measure. Assumes s/c fat is representative of total body fat. Best related to body fat only in the study population.
Body Condition Scoring	+	+	Non-invasive. Easy to perform. Cheap. Fast. No risk to patient.	Best related to body composition in only the study population. Some scorer training helpful.
Ultrasonography	++	++	Relatively non-invasive. Operator training preferable. Relatively fast. No risk to patient. Equipment purchase and maintenance (medium costs). Portable equipment.	Requires relatively expensive equipment. Prone to measurement error (poor probe positioning, intra- and inter-operator variation); possible poor repeatability if exact same sites not imaged. Normally only superficial fat deposits can be imaged, therefore hard to infer about visceral fat. Best related to body fat only in the study population.
Densitometry -under water weighing	+++	+++/+	Unknown.	Requires expensive equipment. Requires patient co-operation/training. Can be lengthy procedure. Assumes density of various body components is constant. Requires correction for residual lung volume and flatus. Unsuitable in animals.
Densitometry -air displacement plethysmography	+++	+++/+	Easily accepted by human patients.	Requires expensive non-portable equipment. Can be lengthy procedure. Assumes density of various body components is constant. Requires corrective assumptions. Unsuitable in animals.
Absorptiometry (photon or X-ray)	++	+++	Relatively non-invasive. Becoming the gold standard for assessment of body	Requires expensive non-portable equipment. Operators must be trained in use of equipment. Animals require

			composition in small animal veterinary practice.	sedation/anaesthesia. Unsuitable in large animals. Can be lengthy procedure. Assumes constant hydration factor for lean tissue.
Total Body Water (indicator dilution)	++ (slightly better for $^{18}\text{O}$ than $^2\text{H}$ )	+++	Minimally invasive.	Assumes indicator evenly distributed throughout all body water and inert. Some indicators are radioactive. Can be expensive (indicator and analysis). Time consuming (equilibrium must be attained). When used to estimate body fat requires assumption of constant hydration factor for lean tissues and that triglyceride content of fat mass is anhydrous. Corrections required for isotope exchange. Usually unquantifiable corrections required for respiratory and urinary losses and effect of gut water. Unpredictable accuracy for estimation of total body water and body fat because validity untested in many species.
Extracellular water	+/+ (depending upon indicator)	++	Minimally invasive.	Assumes indicator evenly distributed throughout only ECF. Some indicators are radioactive. Can be relatively expensive (indicator and analysis). Measured alongside TBW in order to better estimate intracellular fluid and thence body fat, but assumes fat mass is anhydrous.
Total body potassium	+++	+++/+	Non-invasive.	Requires patient co-operation, so unsuitable for animals. Requires expensive equipment. Requires correction for body geometry and background interference. Assumes only lean tissue contains K in estimation of body fat.
Bioelectrical impedance analysis (BIA)	+	+	Relatively non-invasive.	Requires certain degree of patient co-operation (minimal movement). Relies on assumption of constant hydration of lean tissue & anhydrous triglyceride content of fat mass for derivation of fat content. Predictions are specific to the population from which the equations/models were derived. Poor results if patients abnormally hydrated. Poor ability to track changes in fat mass over time. Paucity of validation studies.
Total body electrical conductivity (TOBEC)	+?	+?	Relatively non-invasive.	Expensive equipment. Veterinary patients would require sedation/GA.

**Table 1.2:** Summary of major equine body composition studies. Details of breeds, ages and sexes of animals included in each trial are presented, where available. In addition, body mass (BM), body condition score (BCS) and other measurements recorded are summarised. The term ‘empty body’ differs according to the study, see table for further details where available.

Author/s (year)	Breed	Age	Sex	Number	Live BM (kg)	BCS	Measurements	% Fat
Bradley (1896)	?	Mature?	?	?	Mean 430	?	Visceral and CNS weights compared to live weight	Not recorded
Robb <i>et al.</i> (1972)	Shetland	8mo-18yr	g, m & s	11	81-259	?	Dissection for determination of mass of liver, spleen, kidneys and empty GI tract and its contents. Chemical analysis of empty body (all tissues accounted for except liver). Not exsanguinated.	6.6-18.9% of empty BM (where empty BM = Live BM less contents of GI tract) (Fat determined by ether extraction)
Westervelt <i>et al.</i> (1976)	Ponies	?	?	15 (8 ad lib-fed; 7 limit-fed)	95-150	?mod to fat?	Ultrasound 3 sites s/c fat pre-mortem. Fat depth measured by calliper post-mortem	Used s/c fat depth to determine predictive equations
	Horses?	?	?	8	336-559		Chemical analysis of whole cadaver less gut contents. Method of slaughter and whether exsanguinated was not reported.	15.9±2.0% of empty BM (where empty BM = Live BM less contents of GI tract) (Fat determined by ether extraction)
	Shetland	?	?	11 (6 control; 5 exercised)	?		Chemical analysis of whole cadaver less gut contents. Method of	15.0±0.6% of empty BM (control);

							slaughter and whether exsanguinated was not reported.	9.0±0.8% of empty BM (exercised) Again, empty BM = Live BM less contents of GI tract
Webb and Weaver (1979)	Ponies	2-10yr	4 g 7 m 1 s	12	163-376	8 lean/ poor 3 good 1 fat	*Dissection; visceral weights compared with live weight	Mean 5.1% of live weight (<1 - >11%)
	T'bred	1.5-14yr	1 g 3 m 1 s	5	326-511	4 emac- iated/ poor 1 good		
Martin-Rosset <i>et al.</i> (1983)	Heavy breeds	12-30months	40 colts 35 fillies	75	483 (12mo) to 735 (30mo)	?	*Dissection	Mean 9% (6.7-10.6% depending on age and sex) of empty BM (where empty BM = Live BM less contents of GI tract)
Gunn (1986)	T'bred	?	varied	9	13-490	not debilitated	*Dissection of empty 'carcase' (exsanguinated, possibly eviscerated; not including fats attached to digestive tract). Unclear whether head, tail and lower limbs included.	T'bred: 1.12% of Live BM  Others: 2.11% Live BM
	T-bred X Clydesdale	?		1	109-496			
	Welsh Mt.	?		1				
	Shetland	?		2				
				1				
	T'bred	?		12	0.6-490		Partial dissection; not described.	
	T'bred X Fell Dartmoor	?		3	3-535			
		?		1				
		?		1				



	Conne- mara X Welsh Mt. Shetland	? ? ?		1 3 2				
Kane <i>et al.</i> (1987)	Horses?	1-26yr	varied	6	281-474	good	Ultrasound of rump fat pre- mortem. Chemical analysis of empty body (exsanguinated, eviscerated (incl. Fat), decapitated. Not recorded whether hide, head, tail and lower limbs included.	10-1-24.0% of empty BM (exact definition of empty BM uncertain)
Martin- Rosset <i>et al.</i> (2008)	French Sports breeds	9.8±2.3yr	varied	20	404.5-557.5	BCS 1 to 4.5 (1-5 INRA system)	*Dissection; bomb calorimetry of 'total' soft tissues (fat and muscle)	2.6-14.7% of empty BM (Empty BM= Live BM less contents of GI tract)

g = gelding; m = mare; s = stallion; T'bred = Thoroughbred

\*Dissection was of one 'half' (left or right) of the tissues of the body; whole body values were subsequently calculated assuming equivalence between left and right sides. Where dissection was not completed within 10hr of dissection, corrections were made for 'drip' (Martin-Rosset *et al.* 1983 & 2008).

**Table 1.3:** Overview/summary of techniques used in companion animal species for assessment of body composition.

Species	Objective measure or Index	Measure or Index correlated with:-	Measure or Index suggested as predictor of:-	Reference
Equine	Body mass index (BMI) = $BM (kg)/Withers\ height (m)^2$	BCS	Adiposity	Donaldson <i>et al.</i> 2004  Suggested alternative BMI as (heart girth x length) / withers height Or even heart girth / withers height
Equine	Heart girth/withers height	BCS	Adiposity	Carter <i>et al.</i> (2009a)
	Crest height	Cresty neck score	Regional adiposity	“
	Mid neck circumference / withers height	Cresty neck score	Regional adiposity	“
	Mean neck circumference / withers height	Cresty neck score	Regional adiposity	“
Equine	Ultrasonic rump fat thickness	BCS	Adiposity	Henneke <i>et al.</i> 1983 (using equations of Westervelt <i>et al.</i> 1976)
	Body mass / withers height	Ultrasonic rump fat thickness	Adiposity	“
	Heart girth / withers height	Ultrasonic rump fat thickness	Adiposity	“
Equine	Ultrasonic rump fat thickness	Empty body fat (chemical analysis)	Adiposity	Westervelt <i>et al.</i> 1976  Other sites of subcutaneous fat were also assessed; just caudal to scapula and over ribs
Equine	Ultrasonic rump fat thickness	Decapitated, exsanguinated empty body fat (chemical analysis)	Adiposity	Kane <i>et al.</i> 1987
Equine	Ultrasonic rump fat thickness	Partial empty body fat (no head, lower limbs, skin or GI contents)	Adiposity	Gee <i>et al.</i> 2003  Other sites of subcutaneous fat were also assessed
Feline	Feline BMI = $1.5(ribcage\ circumference\ at\ 9^{th}\ rib - leg\ index\ (patella\ to\ calcaneus)) / 9$	??	Adiposity	Hawthorne and Butterwick 2000
Canine	BMI = Body mass / shoulder height x length (occiput to tailbase)	% fat by DEXA	Adiposity	Mawby <i>et al.</i> 2004
	Other equations including limb length (hock to stifle) and pelvic circumference, some sex-specific	% fat by DEXA	Adiposity	“
Canine	Pelvic circumference	% fat by DEXA	Adiposity	Dobenecker 2008
Canine	Ultrasonic lumbar subcutaneous fat depth	Carcase fat content (ether extraction)	Adiposity	Wilkinson and McEwan 1991  Other subcutaneous fat depots were also assessed

## **1.8 Relevance to equine health and welfare**

Anecdotally, ponies are often said to be more prone to obesity than horses. Whilst obesity is not the sole cause of insulin resistance, greater insulin resistance has been reported among ponies compared with horses, even regardless of their body condition (Jeffcott *et al.*, 1986). This then begs the question of ‘cause or effect’ between greater innate insulin resistance and a possible preponderance towards obesity in ponies. Ponies, especially those of Native breeds, retain a marked seasonality in body condition such that body weight (fat) is gained during spring/summer when food is abundant (which may be helped by greater innate insulin resistance), but is lost during the subsequent winter when forage availability becomes sparse (Fuller *et al.*, 2001; Davies-Morel *et al.*, 2006). It then becomes clear how modern husbandry techniques (year-round access to food (graze or conserved food) of good quality and often in excessive quantity, provision of shelter/housing, use of rugs, minimal work load), combined with global warming (increased winter availability of forage), can promote the summer fat gain, negate the winter fat loss and encourage the development of obesity, especially in ponies.

## **1.9 Objective**

The overall objective of the work was to investigate the application of a horse-specific body condition scoring system in ponies in order to either validate or revise it, or even replace it with a novel system if necessary. This was in order to promote confidence in the application of the subjective technique of body condition scoring for the assessment of adiposity in ponies by veterinarians and owners alike, ultimately to try to facilitate animal monitoring to permit the timely intervention of weight management strategies to address the increasing incidence of obesity among *Equidae*

to improve welfare. To achieve this objective, it was necessary to begin with an investigation of the basic adipobiology in a genetically similar group of animals. Mares from a relatively homogeneous breed of local Native pony, the Welsh Mountain pony, were therefore chosen.

### **1.10 Aims of the thesis**

To achieve this objective, several studies were designed to address specific aspects:-

- Evaluation of the effect of season and body condition on appetite and fattening in Native ponies.
- Determination of precisely where and how much or how little fat can be contained within the pony's body and how this relates to body condition scoring systems.
- Evaluation of methods for measuring body fat content in living ponies.
- Development of feeding and monitoring programmes for the management of controlled weight loss in obese ponies.
- Critique of current body condition scoring systems in the light of our own findings and, if necessary, to facilitate the development of a novel pony-specific BCS system.
  - Field –testing of the novel pony BCS system.

# Chapter 2

## Effects of season and body condition on appetite, body mass and body composition in *ad libitum* fed pony mares

During the writing of this thesis, this chapter has been published:

Dugdale, A.H.A., Curtis, G.C., Cripps, P.J., Harris, P.A., Argo, C.McG. (2010) Effects of season and body condition on appetite, body mass and body composition in *ad libitum* fed pony mares. The Veterinary Journal Doi: 10.1016/j.tvjl.2010.11.009

Preliminary data from this chapter were also presented as an abstract and a poster:

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Dugdale, A., Curtis, G., Cripps, P., Harris, P., Argo, C. (2010) Endogenous seasonal constraints on appetite are insufficient to prevent attainment of obesity in ponies. The Waltham International Nutritional Sciences Symposium: Pet Nutrition – Art or Science? Cambridge, UK, September 2010. P 83.

## 2.1 Summary

Changes in appetite, body mass (BM), body condition score (BCS), direct (ultrasonographic) and indirect (deuterium oxide dilution technique) measures of body fat were monitored in Welsh Mountain pony mares ( $n=11$ , 5-19 years of age) offered *ad libitum* access to a complete diet (Gross Energy  $16.9 \pm 0.07$  MJ/kg dry matter) for 12 weeks during summer ( $n = 6$ ;  $246 \pm 20$  kg) and winter ( $n = 5$ ;  $219 \pm 21$  kg). At the outset, each group comprised two thin (BCS 1-3/9), two moderate (BCS 4-6/9) and two obese (BCS 7-9/9) animals.

For ponies that were non-obese at the outset, BM was gained more rapidly ( $P=0.001$ ) in summer ( $0.8 \pm 0.1$  kg/day) than winter ( $0.6 \pm 0.0$  kg/day). This was associated with a seasonal increase in dry matter intake (DMI) which became maximal (summer,  $4.6 \pm 0.3\%$  BM as DMI/day; winter,  $3.5 \pm 0.1\%$  BM as DMI/day) during the second month. The appetite of the obese ponies was half that reported for non-obese animals in the summer and BM remained constant irrespective of season.

Body 'fatness' increased progressively for non-obese but not obese ponies. Body fat content was exponentially associated with increasing BCS but BCSs  $>6$  were not useful indicators of actual body fat. Endogenous circannual mechanisms to suppress winter weight gain were insufficient to prevent the development of obesity in *ad libitum* fed ponies.

## 2.2 Introduction

Throughout their evolution, ponies native to temperate latitudes adapted to survive in marginal and intensely seasonal mountain and moorland habitats (Speed and Etherington, 1952). Until the last century, these ponies would have been worked within their environment of origin, with little provision of supplementary feedstuffs or shelter (Speed and Etherington, 1952). The past 100 years have seen a progressive increase in the number of these animals being kept under lowland ‘domestic’ conditions. Translocation to such environments has altered nutritional provision from the seasonally variable supply of native grasses to year-round access to highly digestible forages and concentrates. This move has been associated with a dramatically increased incidence of obesity and related disease (Wyse *et al.*, 2008; Argo, 2009).

Ponies, like other herbivores inhabiting temperate latitudes, demonstrate photoperiodically-entrained physiological adaptations which promote survival in an environment where winter forage is sparse (Fuller *et al.*, 2001; Rhind *et al.*, 2002; Thierry *et al.*, 2002; Henry, 2003). In winter, these endogenous circannual rhythms suppress metabolic rate, appetite and body mass/growth and are well documented for many ungulates, including deer (Suttie *et al.*, 1983), sheep (Argo *et al.*, 1999), cattle (Mossberg and Jonsson, 1996) and equidae (Dawson *et al.*, 1945; Argo *et al.*, 1991; Fuller *et al.*, 2001; Scheibe and Streich, 2003). These seasonally-adaptive mechanisms are maintained under domestic conditions, even when access to quality forage is unlimited throughout the year (Rhind *et al.*, 2002). However, the unlimited provision of food and shelter, combined with minimal work, may attenuate the natural tendency of ponies towards winter weight loss. Under natural conditions the body

mass of mature ponies oscillates seasonally around a long term constant or slowly increasing value (Dawson *et al.*, 1945; Scheibe and Streich, 2003). By uncoupling the pony from its natural winter check on body mass gain, domestication may promote year-on-year increments in body mass (BM) leading to obesity and its deleterious effects including the inter-related pathophysiologies of insulin resistance and laminitis (Scheibe and Streich, 2003; Bastard *et al.*, 2006; Geor, 2008). The attainment of obesity in the pony may be hastened by access to pastures comprising high-sugar grass cultivars, developed to meet the extreme metabolic needs of high yielding dairy cattle.

Body fat content is a key participant in the multifactorial regulation of appetite (Forbes, 2003; Woods and D'Alessio, 2008). In humans, appetite is decreased in obese subjects (National Research Council, 1990). Increased body condition scores (BCS) have also been associated with reduced food intakes in sheep and cattle (Bines *et al.*, 1969; Tolkamp *et al.*, 2006).

In ponies, our current understanding of the interactions between the endogenous, seasonally-adaptive controls on appetite and body mass and the regulation of body composition are limited. Further, obesity is rarely encountered in feral ponies. This study tested the hypothesis that 'domestication', exemplified by the provision of shelter, unrestricted access to food of a constant quality and limited exercise, over-rides the endogenous mechanisms which regulate appetite, body mass and composition in the natural environment. To inform the management of native ponies in the domestic state, two relatively homogeneous groups of mature, native-breed mares in diverse outset body condition were observed over 12 weeks in either



winter or summer. By monitoring voluntary food intake (VFI), apparent digestibility of dietary energy, body mass, body condition, body fat content and feeding behaviour, the trial aimed to characterise long-term changes and to evaluate differences associated with season and outset body condition.

## 2.3 Materials and Methods

### *Animals and study design*

All procedures were conducted in accordance with Home Office requirements and were approved by the University of Liverpool's Animal Welfare committee and the Faculty of Veterinary Science's Research Ethics committee.

Two groups of six Welsh Mountain pony mares (5-19yr) were obtained from local pastures (53°N) two weeks prior to the start of the study. One group was studied for 12 weeks during summer (Group S, June to September,  $n = 6$ , age  $10 \pm 2$  yr, outset body mass (BM)  $246 \pm 20$  kg; mean daily temperature  $15.1^{\circ}\text{C}$  [range  $3.8$ - $25.6^{\circ}\text{C}$ ]). The trial was repeated with a separate group of animals during the following winter (Group W, January to April,  $n = 6$ ,  $10 \pm 2$  yr, outset BM  $219 \pm 21$  kg; mean daily temperature,  $7.0^{\circ}\text{C}$  [range  $-4.6$  to  $17.3^{\circ}\text{C}$ ]).

Body condition score (BCS) was determined subjectively by one observer using a 9-point scale from 1 (emaciated) to 9 (obese) as described by Kohnke (1992; modification of Henneke *et al.*, 1983). At the outset of each study, the two seasonal groups contained two 'thin' (BCS 1 to 3), two 'moderate' (BCS 4 to 6) and two 'obese' (BCS 7 to 9) animals. For unrelated reasons, one thin winter pony did not complete the trial and data were excluded from analyses.

Only animals in good general health and dental status were recruited. Routine foot care, vaccination and anthelmintic treatments were provided. From at least one week prior to the start of the study, the animals were individually housed in loose boxes (3m x 4m), bedded on wood shavings over rubber matting and habituated to

handling and measurement protocols. Initial hay feeding was progressively substituted for increasing quantities of the study diet which was available *ad libitum* by the first day of each trial (Day 1). Water was freely available throughout.

Fluorescent strip lighting (220 lux at pony eye level) was controlled by automatic timers to mimic day length at either the summer (Group S, 16L:8D, lights on 06:00 h; lights off 22:00 h) or winter solstice (Group W, 8L:16D, lights on 08:00 h; lights off 16:00 h). Low intensity, red fluorescent lights facilitated animal handling by night. Where possible, ponies exercised freely in a graze-poor paddock for 30min daily.

### ***Nutrition***

The complete chaff-based diet (comprising 25% crude fibre, 8% crude protein, 9.5% ash, ~4 – 5% simple sugars, ~5% starch, gross energy 16.9 MJ/kgDM, fibre length 1 - 3cm; SPILLERS, Milton Keynes, UK), essentially comparable to a moderate quality hay but containing 4% oil, was the sole feedstuff offered in each trial. The diet was provided daily (08:30 - 09:00 h) in deep, anti-spill mangers at floor level in quantities (nearest 10g) calculated to exceed appetite by at least 1 kg. Refused feed was weighed each morning to permit the calculation of voluntary food intake (VFI). For each batch of feed used (Group S, n = 2; Group W, n = 1), three thoroughly-mixed samples (~100g) were collected and stored (-20°C) pending analyses.

### ***Apparent digestibility***

Apparent digestibilities of gross energy (GE), organic matter (OM), dry matter (DM), neutral detergent fibre (NDF), crude protein (CP) and ash in the study diet were determined by total faecal collection over 72h during the first and final weeks of each trial. Faeces were collected immediately after defecation and pooled for each individual in a waterproof sack. Total daily faecal output for each animal was weighed ( $\pm 10$ g, Weigh-Tronix; Avery Weigh-Tronix, West Bromwich, West Midlands, UK), mixed thoroughly and a sample ( $\sim 150$ g) stored ( $-20^{\circ}\text{C}$ ) pending analyses. Each day, fresh food samples ( $\sim 100$ g) were similarly stored. Refused food from each animal was also weighed, sub-sampled ( $\sim 100$ g), stored and subsequently analysed to account for differences in composition between offered and refused feeds.

### ***Feed and faecal analyses***

The DM content of all feed, refused feed and faecal samples was determined after oven-drying ( $70^{\circ}\text{C}$ ) to constant mass. Dried samples were ground (particle size  $< 1$ mm, Moulinex Coffret 5; Groupe SEB Moulinex SAS, Ecully, France) and mixed thoroughly. The GE contents (MJ/kg DM) were determined by bomb calorimetry (E2K Combustion Calorimeter; Digital Data Systems (Pty), Ltd., Northcliff, South Africa). Standard proximate-analytical techniques were used to determine NDF concentrations (Van Soest *et al.*, 1991). Crude protein was estimated by dry combustion (VarioMax CN Macro Elemental Analyser; Elementar Analyse Systeme GmbH, Hanau, Germany) of  $\sim 0.5$ mg (faeces) and  $\sim 0.75$ mg (feed). Sample DM ( $\sim 5$ g) was combusted in a muffle furnace (Carbolite OAF 1; Carbolite Furnaces Ltd., Sheffield, UK), at  $550^{\circ}\text{C}$  to constant mass to evaluate ash content.

### ***Body mass, body condition scoring and morphometrics***

On weekdays (08:30 h to 09:30 h), the BM of each pony was recorded ( $\pm 1$  kg, Lightweight Intermediate weigh scales; HorseWeigh, Llandrindod Wells, UK). Weighbridge calibration was checked monthly against standard weights.

Ponies were body condition scored weekly (Kohnke, 1992) by a single observer blinded to previous records. Heart girth, umbilical belly girth and mid-neck circumference were measured each week ( $\pm 0.5$  cm), with a plasticised measuring tape (We-Bo Animal Measure; Danish Agricultural Association, Copenhagen, Denmark). Measurements were conducted when ponies were relaxed and standing squarely and CVs associated with each procedure were determined using ten repeat measures collected on a single occasion in a single moderate BCS pony.

### ***Ultrasonographic measurement of accessible fat depots***

Each week, the depths of six superficially accessible fat deposits (Table 2.1) were recorded ( $\pm 0.01$  mm) by transcutaneous ultrasonography with a variable frequency (5.5, 7 or 8 MHz) linear array probe (Merlin Ultrasound scanner Type 1101; BK Medical, Herlev, Denmark). Imaging was optimized by clipping the overlying hair and the application of ethanol (70%). Measurements at each site were repeated in triplicate and the mean values were recorded. Ten repeat measurements were made at each site for six ponies of different BCS to enable calculation of median CVs (Table 2.1).

**Table 2.1:** Specific anatomical locations for each of the regional fat deposits evaluated. All measures of fat depth were recorded on the left side of the animal by transcutaneous ultrasonography (variable frequency, 5.5, 7 or 8MHz linear array probe). All measurements with the exception of axillary fat were made in the vertical

<b>Fat deposit</b>	<b>Anatomical location</b>	<b>CV (%)</b>
Tailhead	Probe parallel with vertebral column, immediately lateral to dorsal spinous processes of the sacral/coccygeal vertebrae at the tailhead, just cranial to the first tail hairs.	9.9
Rump	Probe centred on line equidistant between point of hip (tuber coxae) and centre of tailhead (tail hair arc).	10.5
Rib-eye	Probe centred 15cm lateral to dorsal midline in 12 <sup>th</sup> intercostal space.	7.0
Withers	Probe centred equidistant between dorsocaudal angle of scapula and highest point of withers.	9.3
Axillary	Lateral thoracic vein identified at point of emergence between deep pectoral and latissimus dorsi muscles. With lateral thoracic vein in cross section, fat depth immediately adjacent to vein was measured.	19.1
Retroperitoneal	Probe positioned parallel and immediately lateral to ventral midline, just caudal to xiphisternum.	14.7

midline of the image. Coefficients of measurement variation are presented.

### ***Measurement of total body water and estimation of body fat percentage***

Deuterium oxide dilution (D<sub>2</sub>O, 99.8 atom percent excess; CK Gas Products, Hook, Hampshire, UK), was used to measure total body water (TBW) pool size of each pony at the beginning and end of each 12 week study (Fuller *et al.*, 2004). Fat free mass and therefore total body fat mass (TBFM), were subsequently determined by application of the inter-species lean tissue hydration factor, 0.732 (Pace and Rathbun, 1945).

Deuterium oxide doses were individually scaled relative to BM and BCS to account for differences in TBW associated with body fat content (BCS 1 to 4, 0.13g D<sub>2</sub>O/kgBM; BCS 4 to 7, 0.12g D<sub>2</sub>O/kgBM; BCS 7 to 9, 0.11g D<sub>2</sub>O/kgBM), to optimise isotopic enrichments for gas isotope ratio mass spectrometry (Sira 10, VG Isotech, Cheshire, UK). Deuterium enrichments were measured in triplicate for background (immediately prior to D<sub>2</sub>O administration) and equilibration (4 hours following D<sub>2</sub>O administration) samples for each animal as previously described (Wong *et al.*, 1987; Midwood, 1990; Fuller *et al.*, 2004). Data defining TBFM for two, non-ObS animals at the outset of the trial were not available.

### ***Behaviour***

Time-activity budgets were recorded by continuous observation of 4/6 (Group S) and 3/5 (Group W) animals over 24h (09.00 to 09.00h) during the first, 6<sup>th</sup> and 12<sup>th</sup> weeks of each study; a technique previously validated to generate data indicative of typical daily behaviour patterns (Fuller *et al.*, 2001).

Data for each animal were independently recorded (3 observers) as continuous ethograms. Eating, resting (standing and recumbent) and non-eating/non-resting ('play') activities were distinguished. For data analysis, blocks of major behaviours were considered to end or begin when termination or initiation of the behaviour was followed by an interval exceeding 3 minutes of an alternative or continued behaviour.

### ***Clinical biochemistry***

Blood samples (20ml) were collected weekly by venepuncture from each pony, stored and analysed as previously described (Dugdale *et al.*, 2010). Plasma total

protein, albumin, urea, creatinine, triglycerides and total cholesterol concentrations were measured at study start, after the first week and thereafter every month. Plasma glucose concentrations were measured during the first, sixth and final weeks of each trial. Serum concentrations of non-esterified fatty acids (NEFA) were measured weekly. Plasma bile acid concentrations were determined at outset and completion of the study.

Plasma insulin concentrations were measured during the first, sixth and final weeks of each trial using a previously validated ELISA kit (Mercodia Insulin ELISA; Mercodia, Uppsala, Sweden), (McGowan, 2008). Intra-assay CV was 7% (24.1mU/L). Inter-assay CVs were 16.8% (6.0mU/L insulin), 8.7% (24.1mU/L) and 10.0% (53.1mU/L).

### ***Statistical Analyses***

Statistical analyses used Excel (Microsoft Office Professional Edition 2003; Microsoft Corp., Washington, U.S.A.), Minitab version 15.1.0 (Minitab Inc., Pennsylvania, U.S.A.) and STATA 10 (Stata/IC 10.1; Stata Corp., Texas, U.S.A.). Normality of the data was confirmed by assessment of the distribution of residuals using the Anderson-Darling normality test. Statistical significance was assumed when  $P < 0.05$ .

Mixed effects linear regression modelling was performed allowing for both a random intercept and a random component of the change over time (slope), for each pony. The following outcome variables (fixed effects) were investigated: season, BM, BCS and the various linear, circumferential and ultrasonically-derived measurements.



A number of different combinations of season and obesity descriptors were offered as predictor variables including: non-obese (thin and moderate) ponies in summer (non-ObS), non-obese (thin and moderate) ponies in winter (non-ObW) and all obese ponies regardless of season (Ob). A forward stepwise elimination procedure was used to enable selection of the final model. Interactions between variables were investigated where appropriate.

Subjective (BCS), morphometric and ultrasonographic measures were also explored by linear regression and correlation against deuterium oxide dilution-derived measures of body fat.

The apparent digestibilities of each dietary component at the outset and end of each 12-week trial (summer and winter), were compared within and between ponies using general linear modelling with the predictor variables: 'outset' and 'end of trial' and 'group' (non-ObS, non-ObW and Ob).

Blood biochemical variables (total protein, albumin, urea, creatinine, bile acids, triglycerides, cholesterol and NEFA) were analysed by repeated measures ANOVA after appropriate transformation of data where necessary; a Bonferroni-type correction was used for *post hoc* analysis of differences between time points from baseline. Where no significant changes occurred over time, but where differences between ponies according to outset BCS class (Ob or non-Ob) were apparent, data were further explored by Student t-test after appropriate transformation.

Changes in BM are presented as proportions of Day 1 values for clarity. Behavioural data for appetite (in terms of both gDM/kgBM and MJ DE/kgBM) and calculated feeding rates were explored by correlation against proportion of day spent feeding, BM and BCS. All results are presented as mean ( $\pm$  sem) unless otherwise stated.

## 2.4 Results

### *Body mass*

All ponies remained healthy throughout the trials. Examination of the rates of change in BM recorded over 12 weeks suggested that the 11 animals comprised 3 distinct groups with respect to outset BCS and season. Non-obese ponies (thin and moderate BCS at outset) differed between summer (non-ObS,  $n = 4$ ) and winter (non-ObW,  $n = 3$ ) and were distinct from obese animals (Ob,  $n = 4$ ) for which no seasonal influence was determined. Body mass was gained most rapidly by non-ObS ponies and exceeded weight gain in non-ObW animals ( $P = 0.001$ ) by 0.3kg/day (95% confidence intervals: 0.12 to 0.49kg/day) (Fig. 2.1b & Table 2.2).

BM increased substantially for non-ObS ponies over the entire 12-week period (proportional increase,  $0.004 \pm 0.001/\text{day}$ ; actual increase,  $0.8 \pm 0.1\text{kg/day}$ ). This increase was especially pronounced during the first 9 weeks (proportional,  $0.005 \pm 0.001/\text{day}$ ; actual,  $1.0 \pm 0.0\text{kg/day}$ ) of *ad libitum* feeding. Thereafter, the growth of non-ObS animals was checked (Fig. 2.1b). For non-ObW animals, growth was relatively constant over the full 12-week period (proportional,  $0.003 \pm 0.000/\text{day}$ ; actual,  $0.6 \pm 0.0\text{kg/day}$ , Fig. 2.1b). In contrast, the BM of obese ponies did not alter ( $0.2 \pm 0.1\text{kg/day}$ ), irrespective of season (Fig. 2.1b & Table 2.2).

### *Appetite and apparent digestibility of the diet*

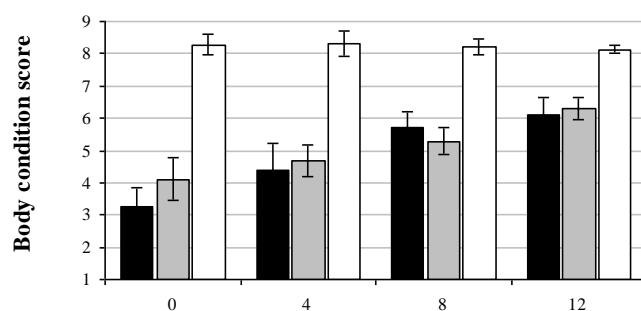
The apparent digestibility of all measured dietary components was constant within- and between-animals irrespective of time, plane of nutrition or outset BCS and data were pooled. Overall, apparent digestibilities were: GE,  $0.50 \pm 0.01$ ; DM,  $0.51 \pm 0.01$ ; OM,  $0.52 \pm 0.01$ ; CP,  $0.62 \pm 0.01$ ; NDF,  $0.38 \pm 0.01$ ; Ash,  $0.43 \pm 0.01$ . The GE

of the study diet was  $16.87 \pm 0.07$  MJ/kgDM. Data describing changes in appetite (gDM/day) were corrected for the apparent digestibility of dietary GE (0.5) and scaled to account for differences in BM (MJ DE/kgBM) to reduce the impact of between-animal differences in absolute body size (Fig. 2.1c).

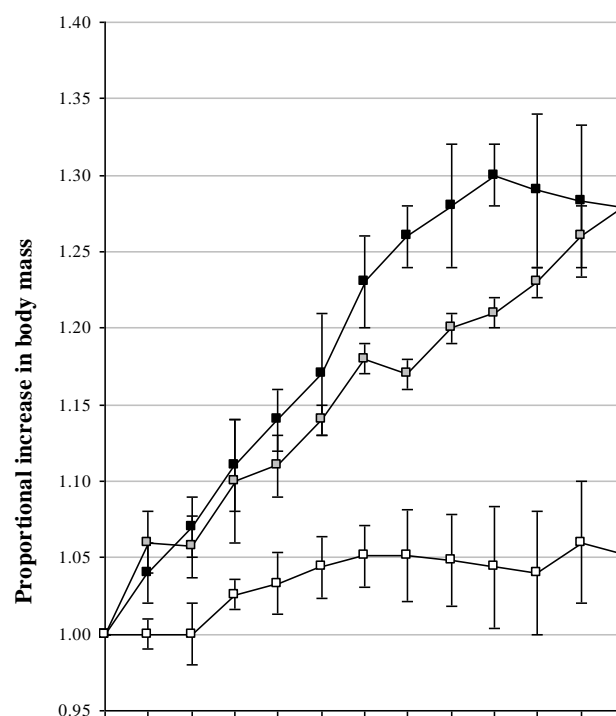
Appetite differed between animal groups and with time (Fig. 2.1c & Table 2.2). Initial appetite was greatest in non-ObS ponies with recorded intakes (scaled for BM) twice those of Ob animals at this time. Appetite increased progressively in non-ObS animals over the first 4 weeks to attain a maximum of  $0.39 \pm 0.02$  MJ DE/kgBM/day ( $4.6 \pm 0.3\%$  of BM as DMI/day). This level of VFI was maintained for 3 weeks before appetite progressively declined. By Week 12, DEI had decreased to  $0.23 \pm 0.04$  MJ DE/kgBM/day ( $2.8 \pm 0.4\%$  of BM DMI/day, Fig. 2.1c). The appetite of non-ObW animals increased more slowly and was maximal after 7 weeks ( $0.29 \pm 0.01$  MJ DE/kgBM/day;  $3.5 \pm 0.1\%$  BM as DMI/day). Thereafter, VFI of non-ObW animals remained relatively constant (Fig. 2.1c).

Food intake of Ob animals was relatively constant with time (overall mean,  $0.17 \pm 0.01$  MJ DE/kgBM/day;  $2.0 \pm 0.1\%$  of BM as DMI). At its greatest, the food intakes of Ob animals (Week 6,  $0.20 \pm 0.1$  MJ DE/kgBM/day;  $2.3 \pm 0.2\%$  of BM as DMI/day) was half that of non-ObS ponies at the same point (Fig. 2.1c). Energy requirements for physiological maintenance (energy balance under the conditions of the trial, including locomotive and digestive costs), determined following regression of DEI on ADG were: non-ObS ponies,  $0.31$  MJ/kgBM/day ( $1.25$  MJ/kgBM<sup>0.75</sup>/day); non-ObW,  $0.26$  MJ/kgBM/day ( $0.98$  MJ/kgBM<sup>0.75</sup>/day); Ob,  $0.16$  MJ/kgBM/day ( $0.68$  MJ/kgBM<sup>0.75</sup>/day).

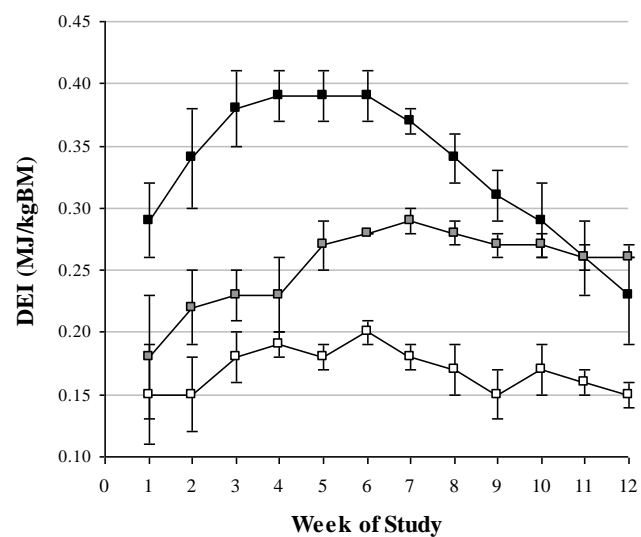
a)



b)



c)



**Figure 2.1:** Mean a) weekly body condition scores b) proportional increases in body mass and c) weekly DEI (MJ/kgBM) for non-obese ponies (thin to moderate outset body condition) in summer (n = 4, black squares) and winter (n = 3, grey squares) and ponies in obese outset body condition in both seasons (n = 4, white squares). Vertical bars denote standard errors of the mean.

**Table 2.2:** Summary of data describing the phenotype of the individual animals at the outset of the study and again, where relevant, on the final measurement date at the end of the 12 week study period. The seasonal group in which each animal was studied is indicated and data for age, height, body mass and body condition score (BCS, scale; 1 = emaciated to 9 = obese) are presented. The percentages of body mass comprised of fat, as determined following deuterium oxide dilution are indicated. Voluntary food intakes are presented for each animal as mean daily dry matter (DM) intakes for the first and last weeks of the study. Voluntary food intakes are also provided during the week at which appetite was maximal (Max) for each individual.

Pony & Season	Age (yrs)	Withers height (cm)	Body mass (kg)		BCS (1 to 9)		Body fat (% BM)		Food intake (kgDM/day)			
			Start	End	Start	End	Start	End	Start	Max	End	
Summer	1	5	116	175	250	2.42	5.58	-	17.01	7.45	11.51	8.71
	2	7	112	271	264	8.50	8.25	18.85	23.86	6.64	7.25	4.17
	3	19	117	208	263	2.17	5.00	4.75	18.54	5.25	11.38	4.30
	4	13	113	314	316	8.67	8.42	30.06	33.33	7.34	8.54	5.11
	5	11	118	270	319	4.59	7.34	11.07	28.15	8.01	14.75	6.69
	6	5	117	236	315	3.84	6.59	-	23.26	9.41	14.06	10.89
Mean ± Std error	10 2.2	115.5 0.99	246 50	287.8 32.0	5.03 1.18	6.86 0.57	16.18 4.44	24.03 2.47	7.35 0.57	11.25 1.21	6.65 1.14	
Winter	A	5	115	196	249	2.75	5.92	7.57	17.02	6.28	8.19	7.60
	B	12	121	293	342	8.58	7.92	27.16	30.47	4.88	8.32	7.58
	D	15	115	239	250	7.34	7.92	21.69	20.32	1.23	5.72	4.02
	E	12	115	189	240	4.84	7.00	11.30	16.13	2.05	7.46	7.30
	F	6	110	176	217	4.75	6.00	11.23	17.13	4.66	6.72	6.34
Mean ± Std error	10 1.8	115.2 1.59	219 20	259.6 19.57	5.65 2.31	6.95 0.40	15.57 3.37	20.21 2.43	3.82 1.56	7.28 2.48	6.57 2.68	

### ***Body condition score***

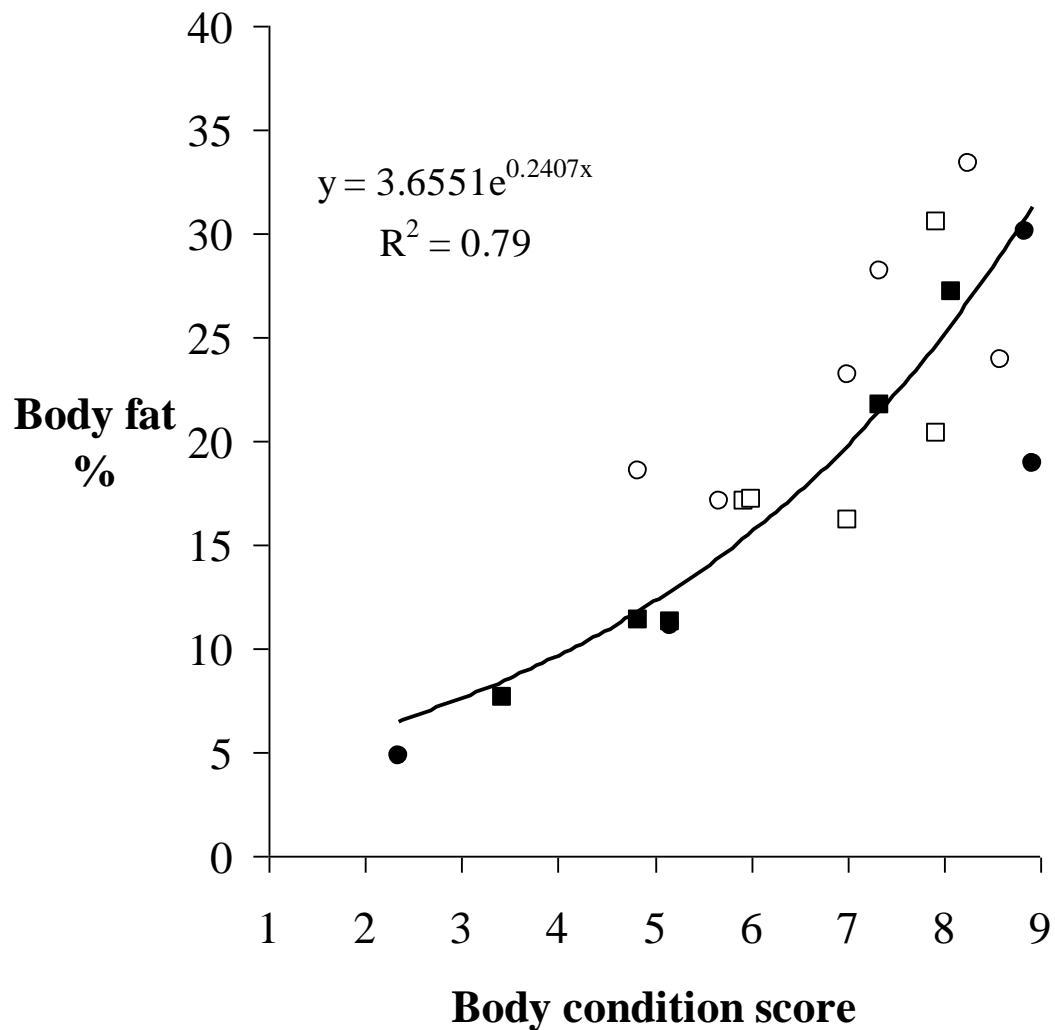
The BCS of ponies in the non-ObS and non-ObW groups increased ( $P < 0.0001$ ) over the study (Fig. 2.1a & Table 2.2). Further modelling of BCS data across non-Ob groups indicated that BCS increased more rapidly ( $P < 0.05$ ) for ‘thin’ (0.27 BCS units/wk, 95% CIs: 0.22-0.33) as opposed to ‘moderate’ animals (0.19 BCS units/wk, 95% CIs: 0.14-0.24). Ninety-one percent of the increment in BCS recorded in the non-ObS group occurred during the first 9 weeks of increased VFI and BM gain (Fig. 2.1). After this, as appetite decreased, BM and BCS gain ceased. Obese ponies demonstrated no significant change in BM, BCS or food intake over the course of the study period (Fig. 2.1 & Table 2.2).

### ***Body fat content***

Regression of BCS on TBFM, expressed as the percentage of BM comprised of fat, for each of the 9 individuals for which the latter data was available at the outset of the study, identified a strong exponential association ( $R^2 = 0.92$ ). However, when data describing the BCS and TBFM of the same animals, 12 weeks later (at the end of both seasonal trials) were included in the regression ( $n = 20$ ), the association was relatively weakened ( $R^2 = 0.79$ , Fig. 2.2 & Table 2.2).

Mean TBFM increased with time for all non-Ob ponies for which outset and end of trial data were available ( $n = 5/7$ , Table 2.2). TBFM gain for non-Ob animals was greatest during summer (non-ObS,  $47.9 \pm 9.5$  kg; non-ObW,  $20.4 \pm 3.3$  kg;  $P < 0.05$ ). The increase in TBFM equalled or exceeded the corresponding gain in BM in non-ObS animals ( $145.2 \pm 59.9$  %). By contrast, fat comprised only half of the BM gained by non-ObW ponies ( $50.2 \pm 8.9$  %). The mean TBFM of Ob ponies increased

(11.5 ±4.3 kg) over the duration of the study, although their BM remained relatively constant (Table 2.2).



**Figure 2.2:** The percentage of body mass comprised of fat, calculated following the determination of body water pool size by deuterium oxide dilution during the first (●, n = 4) and last weeks of the summer (○, n = 6) and first (■, n = 5) and last (□, n = 5) weeks of the winter 12-week studies. Values were regressed on concurrently recorded body condition scores. The solid black line represents the exponential regression across all data points.



### ***Morphometric measures***

Heart girth (HG), umbilical belly girth (BG) and mid-neck circumference (MNC) increased significantly ( $P < 0.0001$ ) in non-Ob ponies in both seasons, but remained constant for Ob ponies (Table 2.3).

Axillary fat depths were highly variable and data were excluded from analysis. All other ultrasonographic measures of fat depths increased over the 12 study weeks in non-Ob ponies ( $P \leq 0.007$ ) (Table 2.3). These changes were not influenced by season or actual outset BCS. For obese ponies, the fat depths remained relatively constant, changing by no more than an average of 0.3mm/wk (Table 2.3).

During the first 9 weeks of the study, when differences in the ‘growth’ rates of the non-Ob groups were most apparent, data suggested that the corresponding rates of change in morphometric and ultrasound markers of fat deposition were seasonally dependent, although most of these associations were not statistically significant (Table 2.4).

Regression of D<sub>2</sub>O dilution-derived estimates of TBFM on contemporaneous linear, circumferential and ultrasonographic measures identified several strong associations which were ranked as potential, non-invasive markers of TBFM (Table 2.4).

**Table 2.3:** Circumferential measurements for 3 body regions and the depth of superficial white adipose reserves determined by trans-cutaneous ultrasound scanning at 5 body regions are provided for data collected at the outset and end of the study. Animals are identified according to the season of study and their body condition score (BCS) classification. Means  $\pm$  standard errors are provided for each group and category of measurement.

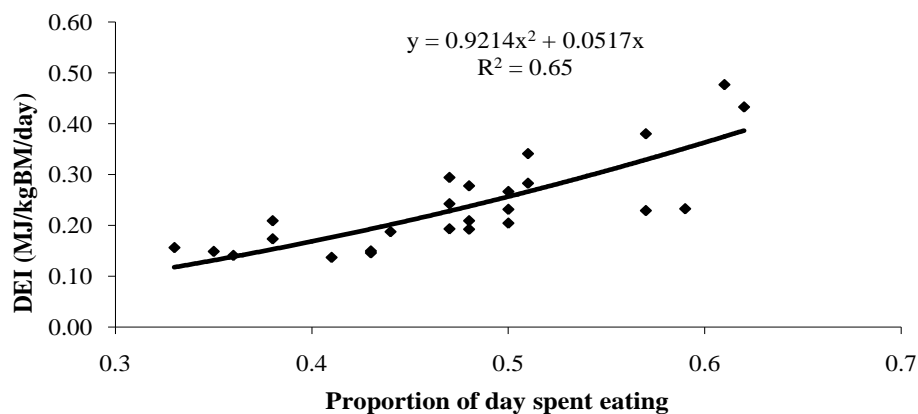
BCS Class	Pony	Circumferential measures (cm)						Depth of superficial white adipose tissues (mm)									
		Heart girth		Belly girth		Mid neck		Tailhead		Rump		Withers		Ribeye		Retroperitoneal	
		Start	End	Start	End	Start	End	Start	End	Start	End	Start	End	Start	End	Start	End
<b>Non-obese Summer</b>	1	137	153	162	175	71	77	7.28	29.57	4.79	11.63	7.70	11.20	0.65	12.43	2.58	29.80
	3	138	148	160	173	73	82	8.39	28.90	10.08	21.20	1.75	11.67	3.95	6.90	3.33	19.37
	5	150	160.5	185	187	73	87	16.93	31.90	4.92	9.85	5.76	11.07	8.83	16.60	8.21	28.20
	6	149	172	177	195	85	92	20.97	31.70	13.2	13.30	5.86	15.30	10.28	19.80	13.63	28.83
		<b>143.5</b> Std error 3.5	<b>158.4</b> 5.2	<b>171.0</b> 6.0	<b>182.5</b> 5.2	<b>75.5</b> 3.2	<b>84.5</b> 3.2	<b>13.4</b> 3.3	<b>30.5</b> 0.8	<b>8.3</b> 2.1	<b>14.0</b> 2.5	<b>5.3</b> 1.3	<b>12.3</b> 1.0	<b>5.9</b> 2.2	<b>13.9</b> 2.8	<b>6.9</b> 2.6	<b>26.6</b> 2.4
<b>Non-obese Winter</b>	A	144	153	157	175	82	87	6.97	22.03	2.92	17.20	0.83	11.77	1.24	10.80	3.47	24.77
	E	140	150	150	167	84	91	8.38	24.37	12.23	28.97	4.88	10.23	6.24	14.77	1.95	7.40
	F	132	151	141.5	170.5	77.5	82.5	16.93	16.63	8.69	14.97	7.01	12.77	10.63	20.63	22.6	40.23
		<b>138.7</b> 3.1	<b>151.3</b> 0.8	<b>149.5</b> 3.9	<b>170.8</b> 2.1	<b>81.2</b> 1.7	<b>86.8</b> 2.1	<b>10.8</b> 2.7	<b>21.0</b> 2.0	<b>8.0</b> 2.4	<b>20.4</b> 3.8	<b>4.2</b> 1.6	<b>11.6</b> 0.6	<b>6.04</b> 2.4	<b>15.4</b> 2.5	<b>9.3</b> 5.8	<b>24.1</b> 8.2
<b>Obese</b>	2	156	155	165	166	85	95	27.90	39.17	18.70	18.97	7.61	10.25	18.17	19.83	21.23	27.53
	4	168	172.5	193	194	81	86	31.27	29.80	17.50	17.93	10.60	11.13	15.57	15.63	27.93	30.00
	B	168	185	184	201	90	99	24.50	29.70	21.60	23.80	6.93	11.40	24.43	30.67	44.37	44.67
	D	153	155	162	167	89	89	14.87	14.13	7.15	9.23	5.87	7.65	12.40	13.07	18.3	11.72
		<b>161.3</b> 3.9	<b>166.9</b> 7.3	<b>176.0</b> 7.5	<b>182.0</b> 9.1	<b>86.3</b> 2.1	<b>92.3</b> 2.9	<b>24.6</b> 3.5	<b>28.2</b> 5.2	<b>16.2</b> 3.2	<b>17.5</b> 3.0	<b>7.8</b> 1.0	<b>10.1</b> 0.9	<b>17.6</b> 2.6	<b>19.8</b> 3.9	<b>28.0</b> 5.8	<b>28.5</b> 6.8

**Table 2.4:** Differences in gains in circumferential body measures (heart girth (HG), umbilical belly girth (BG) and mid-neck circumference (MNC)) and ultrasound generated depths of superficially accessible fat depots for the first 9 weeks of each seasonal study for non-obese ponies (Summer,  $n=4$ ; Winter,  $n=3$ ). The associations between measured variables and total body fat mass (TBFM) over the entire 12 week study for all animals ( $n=11$ ), are presented as correlation coefficients ( $r$ ) with their respective statistical significances ( $P$ ).

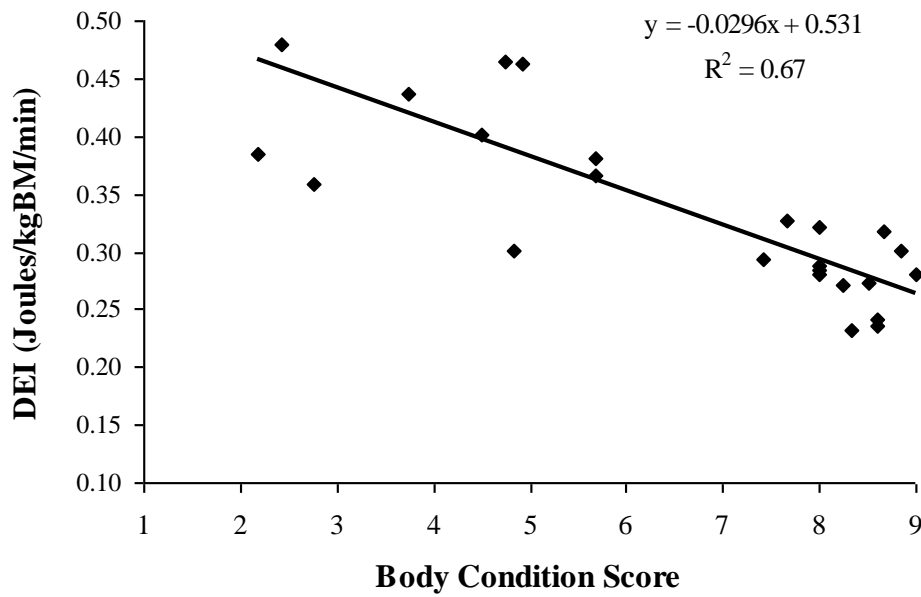
	Variable	Rate of gain			Regression on TBFM	
		Summer	Winter	$P =$	Overall $r$	$P <$
<b>Circumferential measures</b> (cm/wk)	HG	$1.7 \pm 0.3$	$0.9 \pm 0.3$	0.69	0.87	0.001
	BG	$2.7 \pm 0.2$	$0.8 \pm 0.2$	0.20	0.74	0.001
	MNC	$0.8 \pm 0.3$	$1.0 \pm 0.2$	0.31	0.62	0.005
<b>Ultrasound measures</b> (mm/wk)	Rib-eye	$0.6 \pm 0.2$	$0.8 \pm 0.0$	0.53	0.75	0.001
	Tailhead				0.74	0.001
	Rump	$0.7 \pm 0.3$	$1.2 \pm 0.2$	0.27	0.48	0.05
	Withers				0.62	0.005
	Retroperitoneal	$1.3 \pm 0.1$	$0.4 \pm 0.6$	0.59	0.73	0.001

### ***Feeding behaviour***

Irrespective of season and outset BCS, ponies spent approximately half the day feeding (summer,  $0.50 \pm 0.02$ ; winter,  $0.44 \pm 0.02$ ). Feed was consumed as discrete meals, lasting  $40.1 \pm 3.9$  minutes in summer and  $41.9 \pm 3.8$  minutes in winter. As DEI increased, the proportion of the day spent eating also increased; the rate of change was best described by a polynomial function (Fig. 2.3). Across seasons, both total daily DEI and the rate of food consumption (Joules DEI/kgBM/min) were inversely associated with BCS (Fig. 2.4).



**Figure 2.3:** Data describing the relationship between the proportion of the day spent eating and the digestible energy intakes (DEI, MJ/kgBM/day) for ponies of thin and fat outset body condition in summer ( $n = 4$ ) and winter ( $n = 3$ ) for all available animals on each observation occasion ( $n = 25$ ). Data for two fat and one thin animal, on one of each of three observation times were unavailable. The solid black line denotes the polynomial relationship, the equation for which, with its associated coefficient of determination, is presented as a text insert.



**Figure 2.4:** Digestible energy intakes (DEI, Joules/kgBM/min) for ponies of thin or fat outset body condition in summer (n = 4) and winter (n = 3) for all available animals on each observation occasion (n = 25). Data for two fat and one thin animal, on one of each of three observation times were unavailable. The solid black line denotes the linear relationship, the equation for which, with its associated coefficient of determination, is presented as a text insert.

### ***Blood biochemistry***

Mean concentrations for most biochemical variables remained constant and within laboratory reference ranges for all ponies throughout the study (Table 2.5). Although this was true for serum NEFA and plasma triglyceride measurements, Ob ponies had significantly greater concentrations than non-Ob ponies (NEFA, Ob,  $0.22 \pm 0.02$  mmol/L; non-ObS,  $0.13 \pm 0.01$  mmol/L; non-ObW,  $0.10 \pm 0.01$  mmol/L; Triglycerides, Ob,  $0.45 \pm 0.08$  mmol/L; non-ObS,  $0.02 \pm 0.03$  mmol/L; non-ObW,  $0.27 \pm 0.08$  mmol/L). Plasma total cholesterol concentrations increased ( $P < 0.005$ ) progressively and significantly after the second month in all ponies from an overall outset value of  $2.2 \pm 0.1$  mmol/L to attain a mean concentration which slightly

exceeded the reference range (1.5 to 2.9mmol/L) by Week 12 ( $3.1 \pm 0.1$ mmol/L, Table 2.5).

Changes in plasma glucose concentrations were inconsistent (Table 2.5). Overall, mean glucose concentrations were similar between groups and approximated the upper limit of the laboratory reference range (Ob,  $6.4 \pm 0.3$ mmol/L; Non-ObS,  $6.1 \pm 0.3$  mmol/L; non-ObW,  $6.0 \pm 0.5$  mmol/L, Table 2.5).

Plasma insulin concentrations for obese ponies greatly exceeded the published reference range for horses throughout the study ( $209 \pm 53$  mIU/L; reference range, Treiber *et al.*, 2005, 1.22 to 40.40mIU/L, Table 2.5). By contrast, plasma insulin concentrations for all non-obese ponies were much lower ( $P = 0.005$ ) but remained in excess ( $59.2 \pm 11.3$ mIU/L, Table 2.5) of the published reference range for the horse.

**Table 2.5:** Summary of blood biochemistry values for individual animals in the two seasonal study groups. Data for individual animals are presented in accordance with their seasonal group and their body condition score (BCS) classification. Data are presented for plasma concentrations of total protein (TP), albumin, urea, creatinine, bile acids, triglycerides (TG), glucose and insulin. Serum non-esterified fatty acid (NEFA) concentrations are also reported. Where values remained constant across time and within the reference range, values are provided as mean  $\pm$  standard error of the mean of serial samples. Where values for the group or specific BCS categories changed ( $P < 0.05$ ) with time, values for each variable are given at the start (Week 0) and end (Week 12) of the study. Overall group means  $\pm$  standard errors are provided and the reference ranges for each variable are given in the final row.

BCS Class	Pony	Albumin (g/L)	Total protein (g/L)	Urea (mmol/L)	Creatinine ( $\mu$ mol/L)	Bile acids ( $\mu$ mol/L)	TG (mmol/L)	NEFA (mmol/L)	Cholesterol (mmol/L)		Glucose (mmol/L)		Insulin (mIU/L)	
									Start	End	Start	End	Start	End
<b>Non-Obese Summer</b>	1	28.5 $\pm$ 1.8	77.9 $\pm$ 3.6	5.7 $\pm$ 0.6	63.3 $\pm$ 3.6	6.8 $\pm$ 0.1	0.11 $\pm$ 0.02	0.01 $\pm$ 0.01	1.6	2.6	4.8	7.6	8.37	40.82
	3	30.2 $\pm$ 0.8	65.3 $\pm$ 1.6	5.0 $\pm$ 0.4	83.2 $\pm$ 4.1	19.1 $\pm$ 5.4	0.27 $\pm$ 0.04	0.10 $\pm$ 0.00	2.6	3.3	3.9	5.7	11.29	50.58
	5	34.5 $\pm$ 0.6	78.1 $\pm$ 1.3	6.1 $\pm$ 0.5	90.8 $\pm$ 4.5	6.8 $\pm$ 0.7	0.32 $\pm$ 0.06	0.15 $\pm$ 0.01	2.8	3.7	5.4	8.1	30.02	194.15
	6	32.5 $\pm$ 2.1	80.4 $\pm$ 6.7	6.0 $\pm$ 0.1	80.7 $\pm$ 5.5	10.9 $\pm$ 1.7	0.11 $\pm$ 0.03	0.15 $\pm$ 0.01	2.6	3.9	6.5	6.6	84.51	83.74
<b>Overall mean <math>\pm</math> Std error</b>		<b>31.4</b> 0.9	<b>75.4</b> 2.3	<b>5.7</b> 0.2	<b>79.5</b> 3.1	<b>10.9</b> 2.2	<b>0.20</b> 0.03	<b>0.13</b> 0.01	<b>2.4</b> 0.3	<b>3.4</b> 0.3	<b>5.1</b> 0.5	<b>7.0</b> 0.5	<b>33.55</b> 17.65	<b>92.32</b> 35.16
<b>Non-Obese Winter</b>	A	31.2 $\pm$ 2.3	68.0 $\pm$ 2.2	5.3 $\pm$ 0.5	80.6 $\pm$ 5.9	16.3 $\pm$ 1.9	0.17 $\pm$ 0.02	0.09 $\pm$ 0.01	2.2	3.5	6.0	4.5	25.56	29.75
	E	29.6 $\pm$ 0.7	77.4 $\pm$ 4.3	3.5 $\pm$ 0.6	67.9 $\pm$ 2.3	5.4 $\pm$ 3.5	0.44 $\pm$ 0.22	0.12 $\pm$ 0.03	2.3	3.2	5.7	4.5	23.30	48.97
	F	29.8 $\pm$ 2.4	72.3 $\pm$ 5.2	4.6 $\pm$ 0.5	75.8 $\pm$ 3.1	3.5 $\pm$ 1.0	0.18 $\pm$ 0.03	0.09 $\pm$ 0.01	1.7	2.6	9.3	5.0	78.71	15.64
<b>Overall mean <math>\pm</math> Std error</b>		<b>30.2</b> 1.06	<b>72.6</b> 2.4	<b>4.5</b> 0.3	<b>74.8</b> 2.6	<b>8.4</b> 2.7	<b>0.27</b> 0.08	<b>0.10</b> 0.01	<b>2.1</b> 0.2	<b>3.1</b> 0.3	<b>7.0</b> 1.2	<b>4.7</b> 0.2	<b>42.52</b> 18.11	<b>31.45</b> 9.66
<b>Obese</b>	2	32.6 $\pm$ 0.5	70.2 $\pm$ 1.7	4.5 $\pm$ 0.4	87.2 $\pm$ 3.2	4.9 $\pm$ 1.0	0.49 $\pm$ 0.07	0.16 $\pm$ 0.02	2.1	2.4	8.4	7.7	536.22	311.21
	4	36.3 $\pm$ 0.4	75.1 $\pm$ 1.0	5.4 $\pm$ 0.2	101.7 $\pm$ 3.9	10.4 $\pm$ 0.9	0.23 $\pm$ 0.02	0.20 $\pm$ 0.03	2.6	2.9	6.2	7.0	224.53	167.92
	B	31.1 $\pm$ 0.8	59.1 $\pm$ 1.9	4.2 $\pm$ 0.3	80.3 $\pm$ 1.9	5.2 $\pm$ 2.0	0.33 $\pm$ 0.07	0.21 $\pm$ 0.03	1.5	3.0	6.0	4.4	30.7	52.95
	D	26.7 $\pm$ 1.8	69.1 $\pm$ 3.9	3.9 $\pm$ 0.3	70.1 $\pm$ 4.4	6.6 $\pm$ 0.8	0.74 $\pm$ 0.29	0.30 $\pm$ 0.05	2.4	3.3	6.0	4.1	73.01	129.75
<b>Overall mean <math>\pm</math> Std error</b>		<b>31.7</b> 0.9	<b>68.4</b> 1.7	<b>4.5</b> 0.2	<b>84.8</b> 3.1	<b>6.7</b> 1.0	<b>0.45</b> 0.08	<b>0.22</b> 0.02	<b>2.1</b> 0.2	<b>2.9</b> 0.2	<b>6.6</b> 0.6	<b>6.0</b> 0.8	<b>216.12</b> 114.53	<b>165.46</b> 54.15
Reference range		22 - 42	53 - 89	2.3 - 8.7	57 - 123	0.0 - 44.5	0 - 1.0	0.00 - 0.46	1.5 - 2.9		4.1 - 6.6		1.22 - 40.40	

## 2.5 Discussion

This study offers the first evidence that under domestic conditions, with *ad libitum* access to a diet of constant quality, seasonal physiological mechanisms may be insufficient to prevent the excessive accumulation of body fat. Further, with the development of an obese state, the expression of seasonal drives on appetite and BM may be lost.

Irrespective of season, the appetite of animals in thin or moderate outset BCS increased progressively over several weeks. This lag in the attainment of maximal intake may have been dependent on the rate of anatomical adaptation within the gastro-intestinal tract to accommodate the increased DM content of consumed forage (Gross *et al.*, 1985; Rhind *et al.*, 2002). The outset appetites of summer-adapted ponies exceeded those of winter animals by ~65% and this difference was maintained for 6 weeks. It could be inferred that whilst in winter, appetite was constrained by endogenously-generated seasonal-limits, gut capacity may be the dominant factor limiting food intake in summer (Kahn and Spedding, 1984). At its greatest, the DMI of mature non-ObS ponies reached 4.6% of BM. Dry matter intakes of ~5% of BM daily have previously been reported for growing pony colts (Fuller *et al.*, 2001). Current equine nutritional advice, which estimates appetite at around half of these values, should be used guardedly where forage is offered *ad libitum* (National Research Council, 2007).

Appetite may be secondary to underlying changes in energy requirements (Argo *et al.*, 1999, Aiken *et al.*, 1989). Metabolic rate is decreased in winter to minimise energy demands when the quantity and quality of available forage become



limiting (Argo *et al.*, 1991 & 1999; Arnold *et al.*, 2006). Whilst estimates of MER were 16% lower in winter, all ponies remained in positive energy balance throughout. This contrasted with an earlier study where growing colts of the same breed, offered *ad libitum* access to a complete diet of comparable quality, underwent a brief period of negative energy balance at the nadir of their photoperiodically-entrained appetite cycle (Fuller *et al.*, 2001). This may reflect the relatively greater DE requirements of the growing colts or may be indicative of sex-specific responses.

Weight gain in mature animals is generally attributed to the accumulation of body fat. Despite 65% lower DMIs, mature non-ObW animals increased BM by 0.6kg weekly, only 25% less than recorded for non-ObS ponies. As the apparent digestibility of dietary energy was constant between seasons, greater-than-expected weight gain in winter-adapted animals may be a consequence of seasonally-decreased energy requirements for maintenance (Argo *et al.*, 1991; Fuller *et al.*, 2001).

Estimates of TBFM, suggested that for non-ObS animals, body fat content increased more than could be accounted for by the gain in BM alone. Animals in the current study were denied serious exercise. It is possible that weight gain in the absence of exercise may result in the substitution of lost skeletal muscle mass for fat. However, fat comprised only half of the gain in BM for winter-adapted animals. Although the BM of obese animals remained relatively constant, some substitution of lean tissue for fat was also discernable by deuterium analyses although undetected by BCS evaluation. In humans and other animals, increased intramyocellular fat (myosteatorsis) has strong associations with obesity and insulin resistance (Goodpaster and Kelly, 1998; Heilbronn *et al.*, 2004).

Despite relatively increased plasma triglycerides and serum NEFA in obese as opposed to non-obese animals, obese ponies could at no time have been clinically or quantitatively classified as hyperlipaemic. Data describing the association between increased blood triglyceride and NEFA concentrations and body fat content remain inconclusive (Frank *et al.*, 2006; Treiber *et al.*, 2006). The mild increase in plasma cholesterol concentrations noted in all animals may have been associated with the 4% oil content of the study diet (Siciliano and Wood, 1997).

At 2.3% of BM as daily DMI, the appetite of obese animals was half that of maximal values recorded for non-ObS ponies. A similar reduction in the appetite of obese humans and other herbivores has previously been demonstrated but the exact mechanisms mediating this effect have not been confirmed (Bines *et al.*, 1969; National Research Council, 1990, Tolkamp *et al.*, 2006; Woods and D'Alessio, 2008). Data from our study support suggestions that obesity physically constrains gut capacity and decreases metabolic energy requirements (Bines *et al.*, 1969; National Research Council, 1990). Obese animals ate less, more slowly and MERs were approximately halved. The data from non-ObS ponies support the conclusion that appetite suppression is a direct consequence of obesity, as after attaining BCS > 6, their appetite declined progressively to match that of Ob ponies as further gain in BCS and BM ceased, which may have been a consequence of the increased leptin concentrations found in obese individuals (Friedman and Halaas, 1998). These data reinforce the finding of Dugdale *et al.* (2010) that restriction of DMI significantly below 2% was needed to achieve weight reduction in obese ponies. In the present study, appetite and BCS were inversely associated, obese animals spending only a third of the day feeding, half the daily time of ponies in poor BCS.

The MER of obese ponies in this study (0.16 MJ DE/kgBM/day; 0.68 MJ/kgBM<sup>0.75</sup>/day) was very similar to those reported for both Shetland pony stallions and Welsh Mountain pony colts (0.17 MJ/kg/day, Barth *et al.*, 1977; 0.79MJ/kg<sup>0.75</sup>/day, Fuller *et al.*, 2001) and was significantly less than measures from non-obese subjects. Decreased MER has previously been noted in obese cattle (Birnie *et al.*, 2000). For all ponies, MER exceeded current estimates offered by the National Research Council (0.13 to 0.14 MJ/kg/day; NRC, 2007). This may reflect the dominant contribution of horse data over those derived from pony studies within the pooled data (25 studies) appraised by the NRC to derive a composite MER recommendation. In the present study, the overall, physiological MER of stabled, non-obese, mature pony mares was 0.29 MJ DE/kg/day. However, this value oscillated by  $\pm 9\%$  between winter and summer. Previous authors have noted a progressive decrease in the magnitude of the seasonal MER cycle with increasingly domestically-selected sheep breeds (Iason *et al.*, 1994; Argo *et al.*, 1999). In this context, seasonal variation in MER, while possibly insignificant to light horse breeds, may be essential to consider and even breed-specific for Native ponies.

That domestic ponies are more prone to the development of obesity than horses is anecdotally accepted but supporting evidence is sparse. Curiously, although ponies in the current study remained essentially normoglycaemic, all ponies, including those in moderate outset condition, would have been classified hyperinsulinaemic in accordance with reference ranges described for horses (Treiber *et al.*, 2005). These data raise concerns that extrapolation of horse reference ranges for plasma insulin concentrations to the pony may be inappropriate and highlight the need for pony-specific ranges which may be seasonally-dependent. Increased insulin

concentrations have been reported for hibernating species to promote summer fattening (Boswell *et al.*, 1994). It is possible that enhanced food conversion efficiency in the pony is similarly promoted by an increased drive on cellular, as opposed to gastro-intestinal, uptake.

When TBFM was estimated by D<sub>2</sub>O-dilution techniques, fat accounted for between 5 and 33% of BM, values comparable to those reported following the gross chemical analysis of equine cadavers (Robb *et al.*, 1972; Webb and Weaver, 1979). The D<sub>2</sub>O-dilution methodology has been validated against gross chemical analyses for several mammals including dogs and ponies (Burkholder and Thatcher, 1998; Dugdale *et al.*, In Press).

The proportion of total BM estimated to be comprised of fat, increased exponentially as BCS increased ( $R^2 = 0.79$ ). While this BCS system appeared to provide a useful marker of body fat content in ponies of thin to moderate condition (when the sensitivity of the test might be predicted to be greatest over the shallow curvature of the slope), inclusion of data for fatter animals at the end of the study suggested that BCS might be less sensitive for discerning absolute 'fatness' in overweight and obese animals (corresponding to the steeper phase of the curve).

BCS schemes are dependent on progressive changes in the definition of superficial anatomical landmarks as fat is lost or gained (Evans, 1978). For animals at the thin end of the scale, a relatively small change in body fat content is clearly discernible. Conversely, for overweight animals, a much greater change in superficial fat reserves is required to appreciably alter BCS. These data reinforced the relative

insensitivity of BCS protocols as determinants of actual body 'fatness' in overweight animals, a point previously demonstrated during weight loss studies in ponies of the same breed (Dugdale *et al.*, 2010).

All non-invasive measures of body 'fatness', for animals in non-Ob outset condition, progressively increased throughout the studies but greater changes were recorded in summer. The enhanced propensity towards fat deposition in summer-adapted animals confirmed trends described for other ungulates (Rhind *et al.*, 2002; Thiery *et al.*, 2002; Henry, 2003). Closer evaluation of data quantifying markers of subcutaneous fat suggested that this compartment was favoured for fat sequestration in winter. Conversely, there was a tendency for summer-adapted animals to preferentially deposit fat in intra-coelomic compartments. However, the partitioning of fat between different depots is highly variable even in genetically homogeneous populations (Pond, 1998).

Breed, seasonal and individual variation in preferred anatomical sites for fat deposition could potentially confound estimates of body fatness derived from conventional BCS systems which can only assess superficial adipose tissue reserves. Non-invasive methods to appraise intra-coelomic fat are required to complement BCS in the evaluation of total body fat content. Different regional fat depots have been variously implicated in human health and disease (Jensen, 2008). A greater understanding of regional fat deposition in the horse may similarly serve to highlight animals at greater risk of obesity-related disease.

In the current study, changes in heart and belly girths were considered more accurate indices of increasing BM and BCS than mid-neck circumference. Previous studies suggested that subjective appraisal of neck fatness offered a useful proxy for body fat or insulin resistance (Znamiorska, 2005; Frank *et al.*, 2006). In this study, increases in rib, tailhead and retroperitoneal fat depths were closely associated with increments in BCS, while more modest changes were recorded for rump and withers fat. Interestingly, the relative stability of rump fat has previously been reported in earlier studies which monitored weight loss in obese horses and ponies (Gentry *et al.*, 2004; Dugdale *et al.*, 2010). Collectively, these studies would suggest that rump fat deposits are poorly labile.

## **2.6 Conclusions**

Unlike modern horse breeds which have been subject to intense domestic selection, the pony remains a primary product of natural evolution. Naturally-adaptive seasonal changes in appetite, metabolic energy requirements and feeding behaviour persist under domestic conditions. This study would suggest that the physiological drives which promote survival of the pony in the natural-state render these animals unsuited to standard domestic 'equine nutritional protocols'. To optimise health and body condition, domestic management of Native pony breeds should be tailored to match endogenous nutritional expectations in conjunction with careful monitoring of body 'fatness'.

## 2.7 References

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# Chapter 3

## **Effect of dietary restriction on body condition, composition and welfare of overweight and obese pony mares**

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Preliminary data from this chapter were also presented as an abstract:

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### 3.1 Summary

Increased prevalence of obesity among UK horses and ponies demands evidence-based advice to promote weight loss. We hypothesised that restriction of dry matter intake (DMI) to 1% of body mass (BM, 67% of predicted maintenance digestible energy [DE] requirements) would promote weight loss without compromise to health.

Five mature (mean  $\pm$ s.e.  $10 \pm 2$  years), overweight/obese pony mares (BM,  $257 \pm 20$ kg; body condition score (BCS)  $6.8/9 \pm 0.5$ ) were studied over 12 weeks. Animals were individually housed. Daily provision of a chaff-based, complete diet (measured DE, 8.5 MJ/kgDM) was restricted to 1% of actual BM as DMI daily. BCS, girth measurements and ultrasound-derived measures of subcutaneous fat depth overlying the gluteal region and 12<sup>th</sup> intercostal space (rib-eye) were recorded weekly. Body fat content was estimated at the beginning and end of the study by deuterium oxide dilution methods. Clinical biochemistry was monitored weekly. Behaviour was observed (24h, 3/5 ponies) on 3 occasions.

BM decreased by  $4.3 \pm 1.1\%$  during the first week and thereafter by  $0.7 \pm 0.1\%$  of BM at end of Week 1 each week. BCS remained constant. Heart and belly girths, rump width and subcutaneous fat depth at rib-eye decreased significantly with time and BM. Fat comprised  $45 \pm 19\%$  of BM loss. Fatter animals lost relatively more fat. With decreased feeding activity, time spent in 'play' and rest increased by  $36 \pm 11\%$  and  $438 \pm 95\%$  respectively.

This plane of nutrition resulted in an overall rate of weight loss of 1% of outset BM weekly. BCS was not a useful index of early weight loss but heart and belly girths and subcutaneous rib-eye fat were identified as alternative markers.

This study provides an evidence-base for the management of weight loss in obese animals, especially those for which exercise may be contra-indicated.

### 3.2 Introduction

The prevalence of obesity among companion animals in Western civilisations is increasing in line with that observed in humans and has reached approximately 50% in *Equidae* (Sillence *et al.*, 2002; Thatcher *et al.*, 2007; Wyse *et al.*, 2008; Argo, 2009). This may be a direct consequence of domestication and modern husbandry methods (Scheibe and Streich, 2003). In their natural state, *Equidae* express endogenous, seasonally-adaptive changes in appetite and either growth (immature animals) or fat deposition (mature animals), which promote survival and optimise body condition (Fuller *et al.*, 2001). During winter, appetite is decreased and animals enter a period of negative energy balance. Mature animals lose body mass and condition while youngstock suspend growth. Winter losses in body mass and fat content are regained in spring/summer when high quality forage is abundant and young animals demonstrate compensatory growth (Dawson *et al.*, 1945). Under domestic conditions, the provision of shelter and conserved fodder together with relatively low work and thermoregulatory demands, combine to limit natural winter constraints on growth and fat deposition. By facilitating the year-round maintenance of positive energy balance, the effects of successive summer-associated drives on fat deposition are cumulative and may rapidly culminate in the development of obesity (Scheibe and Streich, 2003).

Obesity, defined as ‘excessive body fat content sufficient to cause impairment to health or bodily function’ (NIH, 1985) has been associated with the development of insulin resistance (IR) and a pro-inflammatory state in both man and *Equidae* (Bastard *et al.*, 2006; Treiber *et al.*, 2006; Vick *et al.*, 2007; Geor, 2008). The most frequently encountered and widely recognised consequence of obesity in *Equidae* is laminitis; a

debilitating and painful condition of the feet which often warrants euthanasia. Although the pathophysiology of laminitis is complex, IR appears to play a role (Treiber *et al.*, 2006; Geor, 2008).

Obesity may only be brought to the attention of the owner when acute laminitis occurs; for these animals, the use of exercise as an adjunct to weight loss may be contra-indicated (King and Mansmann, 2004). Although the beneficial effects of weight loss in obese *Equidae* are significant and include increased insulin sensitivity (Van Weyenberg *et al.*, 2008), the effective conduct of dietary restriction is problematic. Sudden and severe restriction of food intake in this species may trigger life-threatening hyperlipaemia (Hughes *et al.*, 2004). Extended periods of confinement without access to forage have been associated with the development of stress-related stomach ulcers and undesirable stereotypic behaviours (McGreevy *et al.*, 1995; Murray and Eichorn, 1996; Redbo *et al.*, 1998; NRC, 2007).

The aims of this study were threefold: to evaluate the weight loss incurred in overweight and obese ponies by a clearly defined, restricted dietary regimen, to devise practical methods for monitoring resultant weight loss and to assess the impact of this weight loss on health and behaviour.

### 3.3 Materials and Methods

#### *Animals and husbandry*

Five mature (5 to 15 year old), Welsh Mountain pony mares  $257 \pm 20$ kg, in overweight to obese body condition score (BCS  $6.8 \pm 0.5$ ; BCS scale: 1 = emaciated to 9 = obese; Henneke *et al.*, 1983; Kohnke, 1992) at the outset of this trial were studied over a 12 week period (April to July). Only animals in good health and dental status were recruited. Routine foot care, vaccination and anthelmintic treatments were administered throughout the study. Ponies were individually housed in loose boxes (3m x 4m) and bedded on wood shavings. Water was freely available. Where possible, ponies were allowed access to a graze-poor paddock for 30min daily to permit social contact. All procedures were conducted in accordance with Home Office requirements.

#### *Study design*

Over the 3 months preceding this study, the ponies had been offered *ad libitum* access to a nutritionally-balanced, chaff-based diet (25% crude fibre, 8% protein, 9.5% ash, 4% oil; SPILLERS<sup>®</sup>, Milton Keynes, UK). For the 12 week duration of this study, dry matter intake (DMI) of the same feedstuff was restricted to 1% of actual body mass (BM) daily. Daily feed provisions (nearest 10g fresh weight) were calculated according to the measured DM of three representative samples of the same batch of study diet (Apparent digestibility section) and were adjusted weekly for individual animals. Daily rations were equally divided between two meals (09:00h and 16:30h) and offered from anti-spill mangers at floor level. All feedstuffs offered were completely consumed by all animals on all days.



### ***Rationale for the selected plane of dietary restriction***

In the current study, DMI was restricted to 1% of actual body mass daily. Based on its actual BM, a hypothetical 250kg obese pony would require 32MJ DE/d (0.127 MJ/kgBM/d (NRC, 2007)). Assuming that 25% of this BM is comprised of excess, relatively metabolically-inert, white adipose tissue, then only 75% of actual BM ‘warrants feeding’, predicting a daily maintenance requirement of 24MJ DE. Provision of 1% of actual BM of the study diet as daily DM would provide 21MJ of DE (GE 17 MJ/kgDM; GE digestibility 0.5). A daily deficit of between 8 and 10.5 MJ DE was therefore predicted. In cattle, each kg of body mass loss yields 28 MJ of ME (Moe *et al.*, 1971). Without consideration of disparate interspecies losses of DE as urine or methane, the hypothetical pony was predicted to lose between 0.3 and 0.4kg daily (0.8 to 1.1% BM, weekly).

### ***Physical measurements***

Ponies were habituated to routine measurement procedures prior to the onset of this study. The BM of each pony was recorded (nearest kg) between 08:30h and 09:30h on the first day each week (Lightweight Intermediate weigh scales; HorseWeigh, Llandrindod Wells, UK). Weighbridge calibration was regularly checked.

### ***Body condition scoring, morphometric and ultrasonographic measurements***

Each week, a series of subjective and objective physical measurements were recorded for each animal. All ponies and measures were assessed in the same order by the same observer (blinded to previous measures) on each occasion. The BCS of each animal was recorded in accordance with criteria described by Kohnke (1992,

modification of Henneke *et al.*, 1983). Linear and circumferential body measures (to the nearest 0.5cm), were made using a plasticised measuring tape (We-Bo Animal Measure, Danish Agricultural Association). Measurements (Table 3.1) were conducted when ponies were relaxed and standing squarely and CVs were determined by repeat measures (n = 10) on a single occasion.

The depth of two superficially accessible fat deposits ('rump' and 'rib-eye'), were measured to the nearest 0.01mm by transcutaneous ultrasonography with a variable frequency (5.5, 7 or 8MHz) linear array probe (Merlin Ultrasound scanner Type 1101; BK Medical, Herlev, Denmark). Measures were repeated in triplicate and mean values recorded. Subcutaneous fat was measured when the probe was either centred over the middle gluteal muscle (equidistant between left point of hip and centre of tail-head root; 'rump'), or positioned parallel with the 12<sup>th</sup> intercostal space, (centred 15cm lateral to dorsal midline; 'rib-eye'). Coefficients of variation were 'rump', 2.8-16.4%; and 'rib-eye' 1.9-13.4%.

**Table 3.1:** Specific anatomical sites used to obtain morphometric measurements which were retrospectively associated with changes in body mass over the 12-week study.

<b>Linear or Circumferential measure</b>	<b>Detailed description of measurement sites</b>	<b>Coefficient of Variation</b>
Rump width	Point of left hip (tuber coxae) to point of right hip, measured over the contour of the dorsum.	1%
Heart girth	Measured during the end expiratory pause, caudal to the points of the elbows, and immediately behind the caudal extremity of the withers.	0.1%
Belly girth	Measured during the end expiratory pause, at the widest point of the belly: approximately 2/3 of the linear distance between the point of the left shoulder and the point of the left hip.	0.2%
Neck circumference at mid-crest	Measured perpendicularly to the top line of the crest, approximately 1/2 of the crest length caudal to the poll; approximately at the position of cervical vertebrae 3 / 4.	0.8%

### ***Measurement of total body water and estimation of body fat percentage***

Total body water (TBW) pool sizes were calculated for each pony at the beginning and end of the 12 week study using deuterium oxide (D<sub>2</sub>O, 99.8 atom percent excess, CK Gas Products, Hook, Hampshire, UK) dilution methodologies previously described by Fuller *et al.*, 2004. Fat free mass and, by association, total body fat mass (TBFM) were subsequently estimated by application of the inter-species lean tissue hydration factor, 0.732 (Pace and Rathbun, 1945).

Deuterium oxide doses were individually scaled relative to BM and BCS to account for differences in tissue hydration associated with body fat content (BCS 7 to 9, 0.11g D<sub>2</sub>O/kgBM; BCS 4 to 7, 0.12g D<sub>2</sub>O/kgBM) to optimise isotopic enrichments for gas isotope ratio mass spectrometry (Sira 10, VG Isotech, Cheshire, UK). Deuterium enrichments were determined in triplicate for each sample as previously described (Wong *et al.*, 1987; Midwood, 1990; Fuller *et al.*, 2004).

### ***Clinical biochemistry***

Blood samples (20ml) were collected weekly by venepuncture from each pony into plain and heparinised tubes (BD Vacutainer; Becton, Dickinson and Co., New Jersey, USA) between 08:30 and 09:00h. Samples were centrifuged immediately (plasma), or after 2h (serum) at 2000g for 10min at 4°C (Hermle Z 300 K; Hermle Labortechnik GmbH, Wehingen, Germany). Plasma / serum samples were stored at -20°C pending measurement of plasma total protein, albumin, urea, creatinine, triglycerides, total cholesterol and glucose concentrations (KoneLab 20i; Thermo Fisher Scientific Oy, Vantaa, Finland), and serum concentrations of non-esterified fatty acids (NEFA), (NEFA-HR(2); Wako Chemicals GmbH, Neuss, Germany).

Serum  $\beta$ -hydroxybutyrate concentrations were measured (Veterinary Laboratories Agency, Shrewsbury, U.K.) at outset, after 1 week and at the conclusion of the study (Ranbut; Randox Laboratories, County Antrim, U.K. and Olympus AU 400 Chemistry Analyser; Olympus, Tokyo, Japan). Plasma bile acid concentrations were determined at outset and completion of the study (KoneLab 20i; Thermo Fisher Scientific Oy, Vantaa, Finland).

Samples were analysed in duplicate. Inter- and intra- assay CVs were calculated from repeated analysis (x10) of equine plasma from healthy individuals. The respective intra- and inter- assay CVs were as follows: glucose, 1% & 4%; albumin, 2% & 3%; total protein 1% & 2%; urea, 7% and 7%; creatinine, 8% & 8%; cholesterol, 1% & 3%; triglyceride, 1% & 4%; NEFA, 2% & 6%. The intra-assay CV for bile acids was 7%. Data describing assay variation for the commercial  $\beta$ -hydroxybutyrate assay were not available.

### ***Insulin ELISA***

Plasma insulin concentrations were measured at the outset of the study and in Weeks 6 and 12 using a commercially available ELISA kit (Mercodia Insulin ELISA; Mercodia, Uppsala, Sweden) previously validated for *Equidae* (McGowan *et al.*, 2008). Intra-assay CV was 7% (24.1mU/L). Inter-assay CVs were 16.8% (6.0mU/L insulin), 8.7% (24.1mU/L) and 10.0% (53.1mU/L).

### ***Behaviour***

Time-activity budgets were determined following continuous observation of 3 of the 5 animals over 24h (09.00 to 09.00h) during the first, 6<sup>th</sup> and 12<sup>th</sup> weeks of the

study; a technique previously validated to generate data indicative of typical daily behaviour patterns (Fuller *et al.*, 2001). Ponies were habituated to the presence of observers on an elevated (2m) viewing platform which offered clear sight of each pen's interior. Observers (n = 3) manually catalogued the behaviours of each pony, independently, against time, in the form of continuous ethograms. Three major behavioural categories were recognised: eating, resting (standing and recumbent) and non-eating/non-resting ('play'). For data analysis, blocks of major behaviours were considered to end or begin only when termination or initiation of the behaviour was followed by an interval exceeding 3 minutes of an alternative or continued behaviour.

### ***Apparent digestibility***

Apparent digestibilities of GE, organic matter (OM), DM, neutral detergent fibre (NDF), crude protein (CP) and ash in the study diet were determined by total faecal collection over 72h during the final week of *ad libitum* feed provision immediately preceding the start of the current study and again after 12 weeks of dietary restriction (Fuller *et al.*, 2001). Refused food (collected during the *ad libitum* fed trial) was weighed ( $\pm 10$ g), sampled and frozen ( $-20^{\circ}\text{C}$ ) for later analysis to permit the calculation of daily DMI.

The DM content of food and faecal samples was determined after oven-drying ( $70^{\circ}\text{C}$ ) to constant mass. The GE content (MJ/kg DM) of all samples was determined by isothermal bomb calorimetry (E2K Combustion Calorimeter; Digital Data Systems (Pty), Ltd., Northcliff, South Africa). Standard proximate-analytical techniques were used to determine NDF concentrations in sample DM (Van Soest *et al.*, 1991). Crude protein contents of sample DM were estimated by dry combustion (VarioMax CN

Macro Elemental Analyser; Elementar Analyse Systeme GmbH, Hanau, Germany) of ~0.5mg (faeces) and ~0.75mg (feed). Sample DM (~5g) was combusted in a muffle furnace (Carbolite OAF 1; Carbolite Furnaces Ltd., Sheffield, UK), at 550°C to constant mass to enable the measurement of ash content.

### *Statistical analyses*

Data were entered into an Excel spreadsheet (Microsoft Office Professional Edition 2003; Microsoft Corp., Washington, U.S.A.) and statistical analyses were performed using Excel, Minitab version 15.1.0 (Minitab Inc., Pennsylvania, U.S.A.) and STATA 10 (Stata/IC 10.1; Stata Corp., Texas, U.S.A.). Log transformation of the data was performed when necessary to improve the validity of model assumptions.

General linear modelling was used to investigate the effects of the predictor variables: pony and time (week) on the outcome variables: body mass, body condition score and the various linear, circumferential and ultrasonically-derived measurements. Time was included as a covariate. (Including pony as a random effect did not alter the conclusions; these results are not presented).

Changes in body condition score and all the linear, circumferential and ultrasonic measurements were also investigated by correlation and least squares linear regression against changes in body mass and time.

After confirming normality of the data, paired t-tests were used to compare the apparent digestibility of the various dietary components at the outset and end of the study. All biochemical variables were analysed by repeated measures ANOVA; a

Bonferroni-type correction was used to determine significant differences of given weeks from baseline.

Changes in physical measurements e.g. BM, are presented as proportions of Day 0 values for clarity. Results are presented as mean ( $\pm$  sem) unless otherwise stated. Statistical significance was assumed when  $P < 0.05$ .



### 3.4 Results

#### *Physical measurements*

Overall food intake decreased from  $6.9 \pm 0.5$  kg DM/day ( $2.7 \pm 0.2\%$  BM as DMI) during *ad libitum* access to food to  $2.7 \pm 0.2$  kg DM/day ( $1.0 \pm 0.0\%$  BM as DMI) in the first week of the study. By the 12<sup>th</sup> week of the study, food intake had reduced further to a mean of  $2.3 \pm 0.2$  kg DM/day ( $1.0 \pm 0.0\%$  BM as DMI). Body mass decreased progressively in all animals ( $P < 0.005$ ) throughout the study. Mean BM decreased by  $4.3 \pm 1.1\%$  in the first week of dietary restriction. From one week after the imposition of the restricted food intake regimen, BM decreased in a linear manner over the remainder of the study at a mean weekly rate of  $0.7 \pm 0.1\%$  of the BM value recorded at the end of Week 1 (Figure 3.1a). The rate of weight loss was least for the most obese animal (Pony B, BCS 8).

Despite considerable losses in BM ( $11.4 \pm 1.9\%$ ; or  $28.9 \pm 3.5$  kg), corresponding changes in body condition score (BCS) were disproportionately small or absent over the 12-week period (Table 3.2). Numerically, BCS decreased ( $P < 0.05$ ) by 0.01 to 0.06 BCS points weekly. However, there was considerable variation in the rates of change between ponies, with an overall mean loss of only  $0.3 \pm 0.14$  BCS points over the 12-week period (Figure 3.1b).

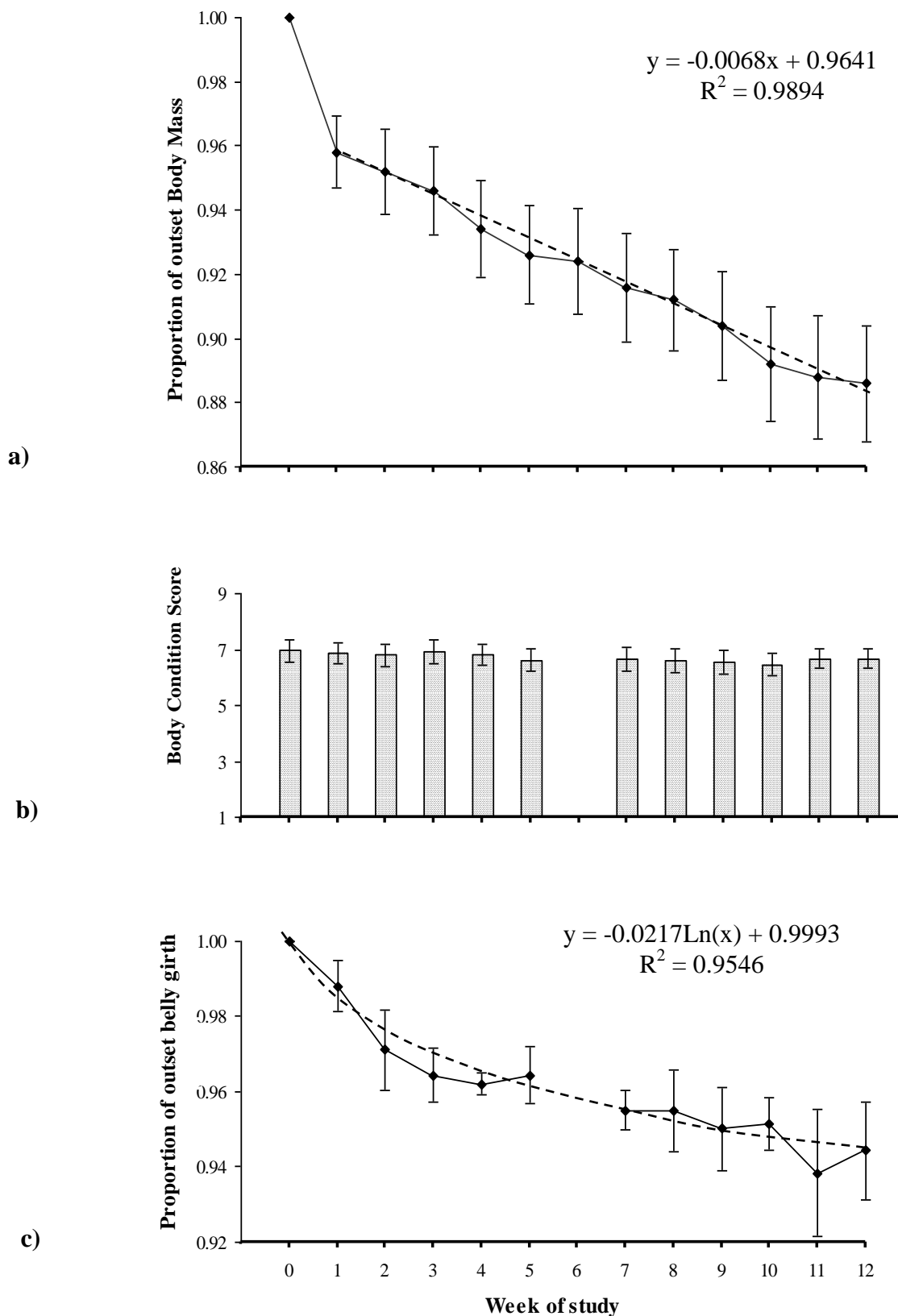
Weekly changes in morphometric measurements were rigorously screened to identify variables which demonstrated strong linear correlations with time and BM both between and within ponies. Body mass reduction was associated with changes in: heart girth ( $r = 0.84$ ), belly girth ( $r = 0.88$ ), rump width ( $r = 0.95$ ), and ultrasound generated measures of subcutaneous fat depth at the rib-eye area ( $r = 0.98$ ). Neck

circumference and rump fat depths did not change consistently as BM decreased (data not presented).

Heart girth decreased ( $P < 0.005$ ) throughout the 12-week period. During the first week of dietary restriction, it decreased by an average of  $3 \pm 2\%$ , thereafter mean heart girth values decreased linearly by  $0.35 \pm 0.1\%$  per week. Mean belly girth also decreased with time ( $P < 0.005$ ) but the pattern of change was more appropriately described by a logarithmic function (Figure 3.1c). The first week of dietary restriction was associated with a mean decrease in belly girth of  $1 \pm 2\%$ .

**Table 3.2:** Data describing changes in body condition score (BCS), body mass (BM) and body composition determined following measurement of deuterium oxide dilution throughout the total body water pool and the relative distribution of weight loss between fat and lean tissues over the 12-week study. Total losses of body fat are expressed as a percentage of total body mass lost.

Pony	BCS		Body Mass (kg)		% Body Fat		Calculated Loss (kg)		Fat loss as % of BM loss
	Outset	Finish	Outset	Loss	Outset	Finish	Fat	Lean	
<b>A</b>	6.1	5.6	254	38	17	15	10	27	28.9
<b>B</b>	8	7.6	342	19	30.5	26	21	-1	105.3
<b>D</b>	7.7	7.1	256	31	20	13	22	9	71.0
<b>E</b>	6.9	6.7	243	43	16	19	1	42	2.3
<b>F</b>	6.1	6.3	221	35	17	17	6	29	17.1

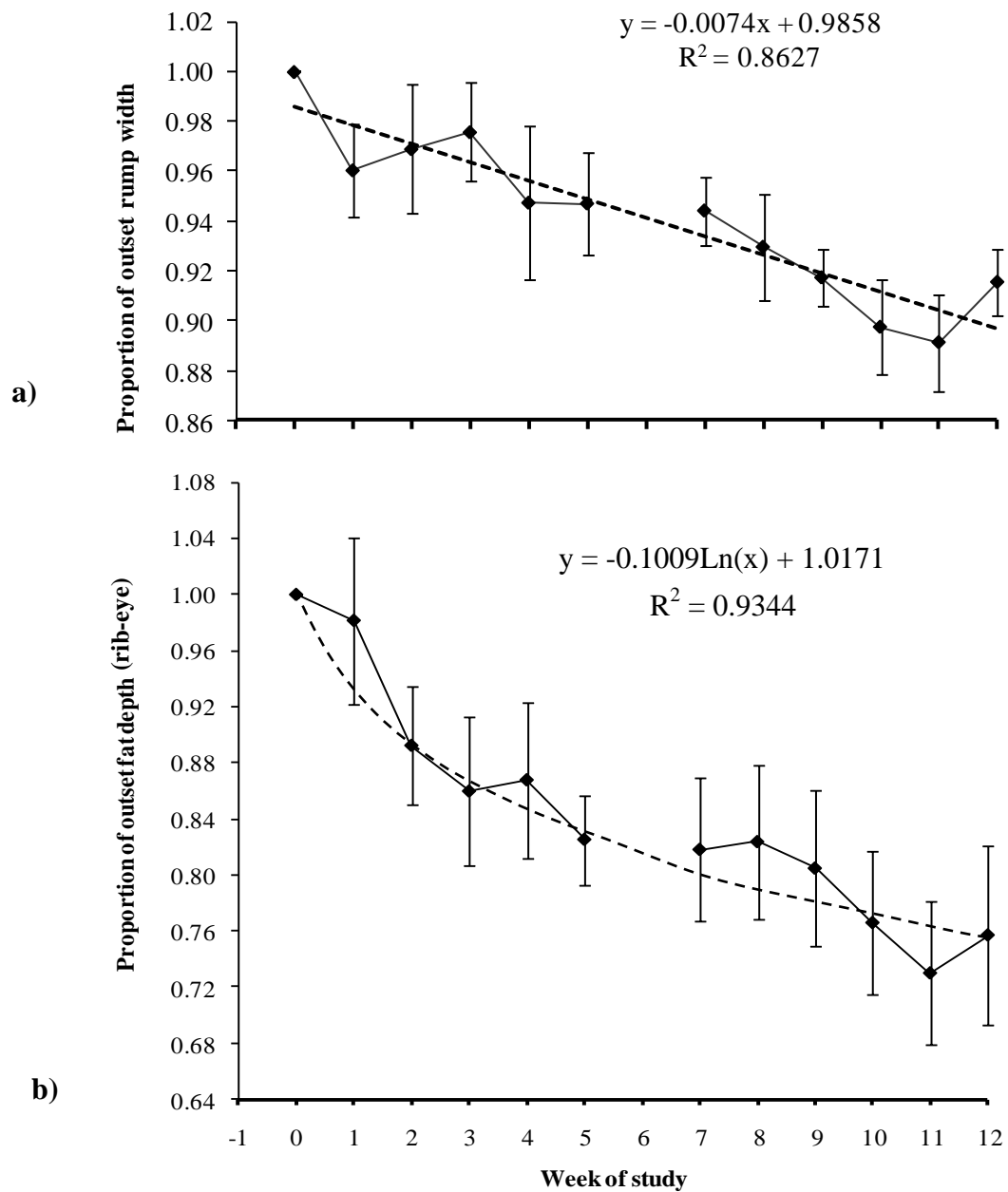


**Figure 3.1:** Mean changes in **a)** the proportion of outset body mass, **b)** body condition score and **c)** the proportion of outset belly girth, are presented for the 5 ponies during the 12-week study (Week 1 = baseline). Equations describing relative changes in BM and belly girth over time are presented with their coefficients of determination ( $R^2$ ). Vertical bars denote the standard error of the mean.

Overall, rump width decreased in a linear manner by  $0.7 \pm 0.1\%$  weekly ( $P < 0.001$ ; 0.2 to 0.6cm/wk, Figure 3.2a). When all 5 ponies were considered, no consistent change in rump fat thickness was observed ( $P > 0.1$ ). However, while rump fat decreased in four ponies ( $2 \pm 0.3\%$  per week;  $\sim 0.3 \pm 0.06\text{mm/wk}$ ), it increased in the most obese animal (by 1% or  $\sim 0.3\text{mm}$  weekly). In contrast, the layer of subcutaneous fat in the rib-eye region decreased logarithmically with time ( $P < 0.01$ ) for all ponies (Figure 3.2b).

### ***Body fat estimation by deuterium oxide dilution***

Although all ponies were overweight or obese at the outset of the study, body fat content was markedly influenced by relatively minor differences in BCS. Almost one third of the BM of the most obese animal (Pony B, BCS 8) was fat-associated; approximately double the fat content of overweight animals e.g. Ponies A, E and F (BCS 6.1 to 6.9). Overall ( $n = 5$ ), fat comprised  $45 \pm 19\%$  of lost BM. The fat content of lost BM was strongly associated ( $R^2 = 0.72$ ) with outset BCS; fatter animals losing relatively more fat (Initial BCS =  $[0.0167 \times \% \text{BM loss as fat}] + 6.1726$ , Table 3.2).



**Figure 3.2:** Mean ( $\pm$ sem) changes in **a)** the proportion of outset rump width and **b)** the proportion of outset rib-eye fat depth are presented for the 5 ponies during the 12-week study (Week 1 = baseline). The equations which best fit the relative changes over time are presented with their coefficients of determination ( $R^2$ ).

### ***Clinical biochemistry***

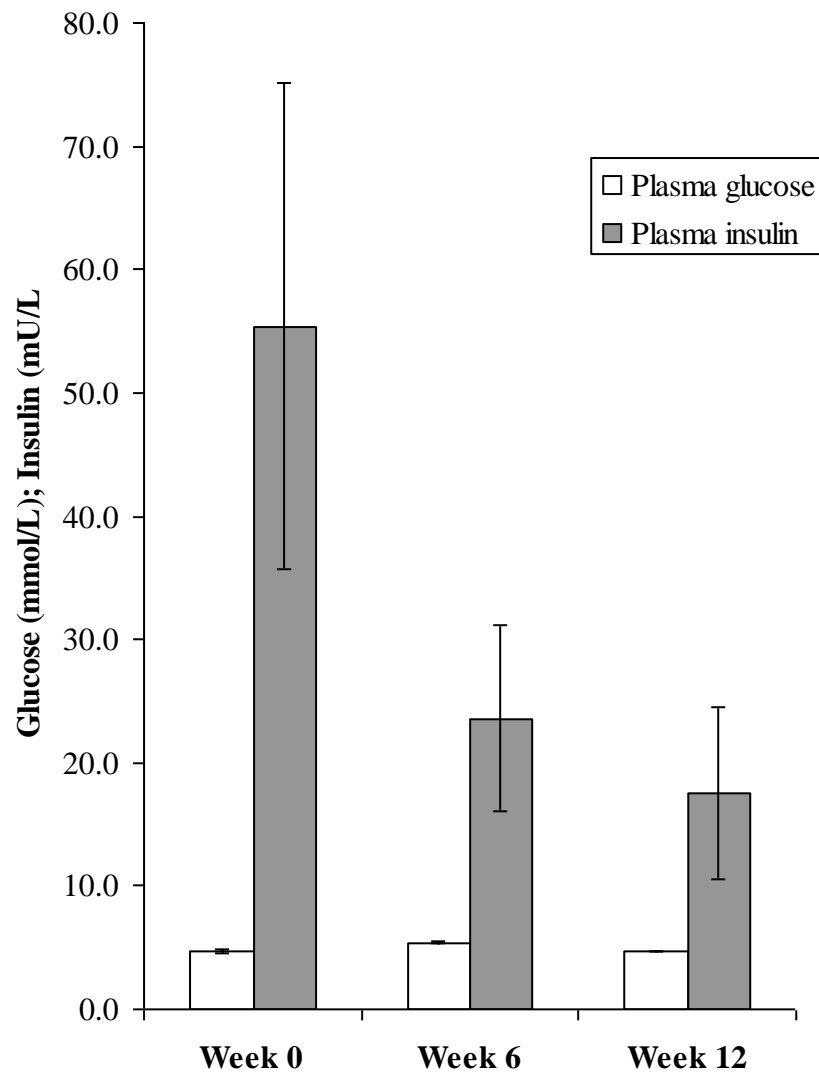
All measured variables remained within the laboratory reference ranges for equine plasma and serum throughout the trial (Table 3.3). Notwithstanding, mean plasma creatinine concentrations increased by almost 50% of outset values ( $68.2 \pm 2.1 \mu\text{mol/L}$ ) over the first month of the study and remained elevated ( $103.4 \pm 0.86 \mu\text{mol/L}$ ) for the remainder of the trial. Mean serum NEFA concentrations increased from  $0.17 \pm 0.04 \text{mmol/L}$  (range 0.05-0.6mmol/L) to  $0.57 \pm 0.08 \text{mmol/L}$  (range 0.1-1.3mmol/L), but plasma triglyceride and total cholesterol concentrations remained unaltered. No evidence of hypertriglyceridaemia or hyperlipaemia was recorded for any animal at any time within the study, either by clinical biochemistry, clinical evaluation or visual inspection of the plasma (Table 3.3).

### ***Plasma glucose and insulin concentrations***

Ponies remained normoglycaemic throughout the study according to our laboratory reference range (4.1-6.6mmol/L), and that of Treiber *et al.*, 2005 (4.1 to 6.9mmol/L) (Figure 3.3). In accordance with the range reported by Treiber *et al.* (2005), i.e. 1.22-40.40mU/L, two ponies remained normoinsulinaemic throughout the study, while the three animals with the greatest BCSs (7, 8 and 8/9) were hyperinsulinaemic, i.e. plasma insulin > 40.4mU/L, (130mU/L, 53mU/L and 49mU/L) at outset. Only one of these remained slightly hyperinsulinaemic (44mU/L) at the mid-point of the study when the other 2 ponies were normoinsulinaemic (35mU/L and 26mU/L). One pony was slightly hyperinsulinaemic again at the end of the study (outset 53mU/L, mid-study 35mU/L, end of study 44mU/L). Regression of change in estimated body fat percentage on change in plasma insulin concentration revealed a weak linear association ( $R^2 = 0.38$ ).

**Table 3.3:** Mean ( $\pm$ sem) weekly concentrations of blood biochemical variables and insulin for the five ponies from one week before the onset of the current trial (Baseline), when animals were *ad libitum* fed and throughout subsequent the 12 successive weeks of restricted feed intake. All analytes were evaluated in plasma with the exception of  $\beta$ -hydroxybutyrate and Non-esterified fatty acids which were determined from serum samples. Values which differed ( $P < 0.05$ ) from Baseline are indicated with an asterisk.

Analyte (Reference range)	Baseline	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12
<b>Albumin</b> (22-42 g/L)	30.8 $\pm 0.6$	31.0 $\pm 0.5$	32.8 $\pm 0.6$	32.8* $\pm 0.8$	30.8 $\pm 0.7$	32.4 $\pm 0.8$	32.8 $\pm 0.6$	33.8* $\pm 0.5$	32.7 $\pm 0.9$	33.0 $\pm 0.6$	34.1* $\pm 0.5$	33.8* $\pm 0.7$	33.8* $\pm 0.3$
<b>Total protein</b> (53-89 g/L)	61.5 $\pm 1.6$	61.9 $\pm 1.7$	67.5* $\pm 1.3$	66.8* $\pm 1.5$	61.3 $\pm 1.5$	64.9 $\pm 2.1$	64.7 $\pm 2.4$	67.6* $\pm 1.7$	64.0 $\pm 1.7$	66.1 $\pm 2.4$	66.0 $\pm 2.4$	66.3 $\pm 2.3$	59.2 $\pm 2.9$
<b>Urea</b> (2.3-8.7 mmol/L)	4.7 $\pm 0.1$	4.6 $\pm 0.4$	3.8* $\pm 0.3$	3.7* $\pm 0.3$	3.5* $\pm 0.2$	3.1* $\pm 0.2$	3.5* $\pm 0.2$	3.3* $\pm 0.3$	3.3* $\pm 0.2$	3.5* $\pm 0.3$	3.5* $\pm 0.2$	3.4* $\pm 0.3$	3.8* $\pm 0.2$
<b>Creatinine</b> (57-123 $\mu$ mol/L)	68.2 $\pm 2.1$	79.5* $\pm 2.1$	88.5* $\pm 2.9$	92.1* $\pm 3.3$	90.4* $\pm 4.0$	96.6* $\pm 4.9$	97.4* $\pm 1.8$	102.1* $\pm 2.2$	101.3* $\pm 2.6$	104.3* $\pm 2.4$	103.4* $\pm 1.9$	105.8* $\pm 2.1$	103.2* $\pm 1.9$
<b>Triglycerides</b> (0-1.0 mmol/L)	0.3 $\pm 0.0$	0.4 $\pm 0.0$	0.4 $\pm 0.1$	0.4 $\pm 0.1$	0.6* $\pm 0.1$	0.4 $\pm 0.1$	0.4 $\pm 0.1$	0.4 $\pm 0.0$	0.4 $\pm 0.1$	0.3 $\pm 0.1$	0.4 $\pm 0.1$	0.4 $\pm 0.1$	0.3 $\pm 0.0$
<b>Total cholesterol</b> (1.5-2.9 mmol/L)	2.7 $\pm 0.1$	2.6 $\pm 0.2$	2.5* $\pm 0.1$	2.5* $\pm 0.2$	2.3 $\pm 0.2$	2.4 $\pm 0.2$	2.4 $\pm 0.3$	2.5 $\pm 0.3$	2.5 $\pm 0.3$	2.4 $\pm 0.3$	2.3 $\pm 0.2$	2.4 $\pm 0.3$	2.3 $\pm 0.3$
<b>Non-esterified fatty acids</b> (0-0.5 mmol/L)	0.17 $\pm 0.04$	0.57* $\pm 0.04$	0.49* $\pm 0.04$	0.47* $\pm 0.04$	0.18 $\pm 0.02$	0.44* $\pm 0.12$	0.74* $\pm 0.16$	0.69* $\pm 0.09$	0.64* $\pm 0.08$	0.60* $\pm 0.08$	0.59* $\pm 0.07$	0.58* $\pm 0.04$	0.57* $\pm 0.08$
<b><math>\beta</math>-hydroxy butyrate</b> (recommended 0-0.96 mmol/L)	0.20 $\pm 0.02$	0.17 $\pm 0.01$											0.16 $\pm 0.01$
<b>Bile acids</b> (0-44.5 $\mu$ mol/L)	7.3 $\pm 1.3$												8.3 $\pm 1.2$
<b>Glucose</b> (4.1-6.6 mmol/L)	4.7 $\pm 0.1$	4.9 $\pm 0.2$	5.4 $\pm 0.2$	5.5* $\pm 0.2$	5.3 $\pm 0.3$	5.8* $\pm 0.3$	5.4* $\pm 0.1$	5.6* $\pm 0.2$	5.5* $\pm 0.2$	5.4* $\pm 0.2$	5.7* $\pm 0.2$	5.8* $\pm 0.2$	4.7 $\pm 0.1$
<b>Insulin</b> (1.22-40.40 mIU/L)	55.41 $\pm 19.77$						23.56 $\pm 7.53$						17.52 $\pm 7.04$



**Figure 3.3:** Mean ( $\pm$ sem) plasma concentrations of glucose (open bars) and insulin (solid bars) measured in blood samples collected at the outset, middle and end of the 12 week period of restricted food provision.



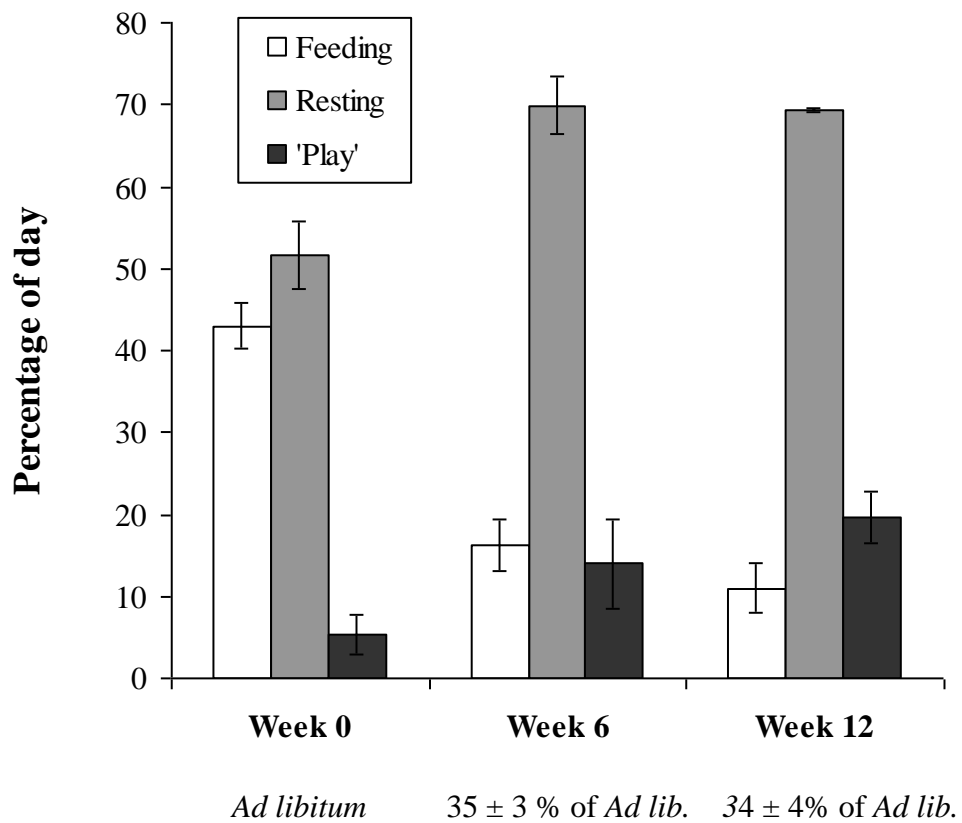
### ***Behavioural changes***

Dietary restriction to  $34 \pm 4\%$  of *ad libitum* intake ( $6.9 \pm 0.5$  kg DM/day *ad libitum* to  $2.3 \pm 0.2$  kg DM/day by Week 12) was associated with a  $75 \pm 5\%$  decrease in daily feeding activity by Week 12. Reduced food intake was associated with decreased daily meal frequencies from  $16.0 \pm 1.2$  during the *ad libitum* fed phase to only  $4.0 \pm 0.6$  meals per day during dietary restriction. However, for limit-fed ponies,  $89.6 \pm 3.3\%$  of the total food allowance was consumed during the first 2 single meals which immediately followed twice-daily food introductions. The mean duration of the meal which followed introduction of half of the daily ration, was  $99.5 \pm 6.2$  min in Week 6 and  $71.2 \pm 13.6$  min in the final week of the study, compared with a mean meal duration of  $40.9 \pm 6.0$  min during the *ad libitum* trial. In addition to an increase in meal duration, the rate of food intake also increased with dietary restriction (Week 0, *ad libitum* fed,  $10.7 \pm 0.6$  gDM/min feeding; Week 6,  $11.7 \pm 1.1$  gDM/min feeding; Week 12,  $17.3 \pm 2.1$  gDM/min feeding). Over the same period, time spent in non-feeding activities ('play') and rest increased by  $437.8 \pm 95.0\%$  and  $35.9 \pm 10.9\%$  of Week 0 (*ad libitum* fed) values respectively (Figure 3.4). One animal, for which behavioural evidence was unavailable, appeared to spend more time walking within the box. Two of the behaviourally monitored ponies displayed increased drinking / water-play activity for which no clinical cause was determined. These unusual behaviours ceased on termination of the study.

### ***Apparent digestibilities***

The apparent digestibility of evaluated dietary components did not alter significantly following 12 weeks of dietary restriction. Data for all animals in both

digestibility trials were pooled and the apparent digestibilities for individual dietary components were GE,  $0.50 \pm 0.01$ ; DM,  $0.52 \pm 0.01$ ; OM,  $0.52 \pm 0.01$ ; CP,  $0.62 \pm 0.01$ ; NDF,  $0.39 \pm 0.01$  and ash  $0.49 \pm 0.01$ . Defecation frequency halved ( $50 \pm 4\%$  of *ad libitum* fed) for all animals with the imposition of restricted feeding and by the end of the study, the average daily output of fresh faeces had decreased to  $35 \pm 8\%$  of that recorded during *ad libitum* feeding. Faecal moisture and the mass of individual defecations remained relatively constant.



**Figure 3.4:** Changes in the mean ( $\pm$ sem;  $n = 3/5$  ponies) proportions of the day spent in feeding, resting and 'play' activities. Data were recorded during the final day of *ad libitum* food provision and again in the 6<sup>th</sup> and 12<sup>th</sup> weeks after the onset of dietary restriction to 1% of body mass as dry matter.

### 3.5 Discussion

Restriction of food intake to 1% of BM as DM daily was associated with a consistent rate of weight loss in all animals from one week after the outset of the 12 week study. Relative weight loss over the first week (-4.3% of outset BM) was 6 fold greater than that recorded subsequently. Enhanced weight loss in the first week most probably reflected decreased gut fill and possibly the depletion of liver and muscle glycogen reserves associated with the transition from *ad libitum* intake (2.6% of BM as DM) to a steady-state of negative energy balance. Overnight fasting (18-20hr) and consequent changes in gut fill have been associated with a 5% decrease in BM in horses and ponies (Webb and Weaver, 1979). In man, loss of muscle glycogen with its attendant water has been associated with a 4.8% decrease in BM recorded after the first 4 days of severe dietary restriction (Kreitzman *et al.*, 1992).

The optimal rate of weight loss for *Equidae* without compromise to overall health is unknown, although on the basis of data in other species, a target loss of 1% BM weekly may be considered appropriate (German *et al.*, 2007). Given that the majority of weight loss in Week 1 was largely a result of physiological adaptation to dietary change, BM at the end of this initial week was a more useful predictor of subsequent weight loss which was consistent and approached target rates.

Published advice for the nutritional management of obese horses begins variously with the restriction of forage intake to 1.3-1.5% of actual or target BM as daily DMI (NRC, 2007). In this study, restriction of intake to 1% BM as DM daily was equivalent to 67% of predicted maintenance requirements based on actual BM (NRC, 2007). A recent study characterised weight loss in obese Shetland ponies (n =

9, unknown sex), limit-fed on a mixture of alfalfa hay and straw (Van Weyenberg *et al.*, 2008). Overall, provision to 51% of estimated maintenance net energy resulted in a mean weight loss of 1% of outset BM weekly, a value comparable to the current study (0.95% of outset BM weekly) calculated on the same basis (Van Weyenberg *et al.*, 2008). Although direct comparison between the two studies is confounded by methodological differences, it would appear that clinically-useful rates of weight loss (~1.0% BM weekly) in obese, Native pony breeds requires that energy intake is limited to between 50 and 70% of maintenance requirements.

Irrespective of the overall loss of  $11.4 \pm 1.9\%$  BM achieved during the current 12 week study, it is important to highlight that for practical purposes, BCS remained constant over this time and did not offer a useful index of weight loss. However, the longer Belgian study attained a further 6.5% decrease in BM over an additional 6 weeks and reported a 4 point decrease in BCS from outset values using a comparable 9 point BCS system (Van Weyenberg *et al.*, 2008).

Repeatability of the BCS system in ponies, between observers and breeds, has not been tested to date. The observation that BCS cannot be used as a reliable monitor of early weight loss, alongside the labour requirements and emotional impact of restricting food intake to companion animals, suggests that owner compliance may be compromised without a measurable index of success.

Of the variables recorded during the current trial, body circumference provided a potentially useful index of weight loss. Initial changes in belly girth probably reflected decreased gut fill following the imposition of dietary restriction in

the first week. Thereafter, it seemed most probable that subsequent changes in belly girth were associated with decreased internal (visceral / retroperitoneal) fat reserves. In man, visceral adiposity has been considered a greater risk factor for IR, morbidity and mortality, than subcutaneous fat (Bergman *et al.*, 2007).

Previously, only marked weight loss associated with a clear decrease in BCS has been demonstrated to improve insulin sensitivity (Freestone *et al.*, 1992; Van Weyenberg *et al.*, 2008). In the current study, comparatively modest weight loss over the first 6 weeks (~6% of outset BM), insufficient to alter BCS, was accompanied by a marked reduction or correction of hyperinsulinaemia (one of the manifestations of compensated IR), in the most obese animals (Geor, 2008). Further, it was noteworthy that data in the current trial were not confounded by a change of diet and suggest that even modest weight loss, achieved in the absence of exercise, can elicit an important improvement in this key risk factor for equine morbidity (Treiber *et al.*, 2006; McGowan, 2008; Schmidt *et al.*, 2009).

Linear regression of heart girth:withers height ratio on basal plasma insulin concentrations was performed after the former measure was determined to be strongly associated with total body fat content (Chapter 4). The relationship between these two variables was, however, poor ( $R^2 = 0.20$ ), possibly due to the small number of observations available. The relationship between change in body fat percentage and change in plasma insulin concentration was weak supporting observations that single determinations of plasma insulin concentration can be misleading (Pratt *et al.*, 2009).

Rump fat depths remained relatively constant throughout this trial, an observation which was in agreement with data reported for horse mares during a 9 month period when BCS was progressively increased (BCS 7 to 8-8.5/9; n=12) or decreased (BCS 7 to 3-3.5/9; n=12) (Gentry *et al.*, 2004). While rump fat depth remained relatively constant in this study, overall rump width decreased by  $8.0 \pm 1.4\%$  over the same time period. Decreases in this measure might provide a generalised index of depletion in all underlying soft tissues, both muscle and fat, resulting from either dietary or exercise restriction. By contrast with other regional fat deposits, and in agreement with Gentry *et al.* (2004), subcutaneous adipose tissue in the 'rib-eye' area decreased consistently with weight loss in all ponies. The subtle changes in subcutaneous fat depths associated with weight loss in this study emphasized the value of palpation as opposed to visual appraisal in the application of any BCS system, especially for animals in winter coat. Nevertheless, the reduction in subcutaneous fat depth, at least at the rib-eye site, made little impact on overall BCS during the 12 week study.

Fat stores within the neck crest offer the most readily appreciated superficial index of adiposity in the pony (Carter *et al.*, 2009). A strong correlation between crest fat depth measured at the level of the 4<sup>th</sup> cervical vertebra and total carcass fat has been recorded in horse cadavers (Znamirowska, 2005). Mean neck circumference or neck crest scoring may provide indirect estimations of IR and whole body adiposity (Znamirowska, 2005; Frank *et al.*, 2006). Although Frank *et al.*'s study (2006) demonstrated a clear association between the magnitude of IR and mean neck circumference, data from this trial suggest that crest scoring systems may be relatively

insensitive indices of early weight loss and the accompanying beneficial changes on IR, at least in Native pony mares.

Estimates of body fat content ranged between 13 and 30.5% of BM for overweight to obese (BCS 5.6 to 8/9) ponies and were comparable with values reported following cadaver dissection or gross chemical analysis (Robb *et al.*, 1972; Webb and Weaver, 1979). The estimation of total body fat mass (TBFM), following determination of TBW pool sizes by deuterium oxide dilution, has been reported for several mammalian species including man and dogs (Schloerb *et al.*, 1950; Burkholder and Thatcher, 1998). To date, estimation of TBW by deuterium oxide dilution has been reported for use in *Equidae* (Andrews *et al.*, 1997) but the association between deuterium-derived values and gross physical or chemical cadaver composition has not been confirmed in this species. Equilibration of administered deuterium oxide within the relatively substantial digesta compartment of large herbivores might comprise an important source of error (Sneddon and Argenzio, 1998). In the current study, BM-scaled intake of a common feedstuff would have minimised errors arising from this source in data derived during the final week of the study. However, the relatively greater and more varied gut fills of the *ad libitum* fed ponies, at the outset of the study, could have potentially contributed to a relative underestimation of TBFM at this time, thus minimising the overall derived change in TBFM.

It was notable that a one or two point increase in BCS (between 6 to 6.9 and 8) was associated with a doubling of body fat content. That such dramatic fat deposition is associated with only relatively modest changes in BCS raises an important issue.



Linearly ordinal BCSs may be relatively insensitive tools for monitoring changes in body composition in overweight / obese animals. This would agree with findings in cattle which suggested that linear increments in BCS were associated with a curvilinear increase in body fat content (Gregory *et al.*, 1998). BCS systems are dependent on the appraisal of superficial (external) fat deposits alone and cannot reliably detect increased internal adiposity. Further, when subcutaneous, inter- and intra-muscular adipose tissues have developed sufficiently to obscure key anatomical landmarks, only dramatic further expansion of these reserves can perceptibly alter external appraisal.

Total weight loss in the most obese pony was only 5.6% BM, all of which was apparently fat. This contrasted markedly with BM loss in the other animals in which tissue mobilisation was distributed variously between fat and lean tissues. The proportion of weight loss which comprised fat was clearly associated ( $R^2 = 0.72$ ) with outset BCS. Previous workers have suggested a similar bias towards fat mobilisation in fatter human subjects and hibernating species (Forbes, 2000). Fat mobilisation was discernable as increased serum NEFA concentrations (Frank *et al.*, 2002). However, evidence of hyperlipaemia was absent in this and Van Weyenberg *et al.*'s (2008) study, which may promote confidence in the application of comparable dietary restriction protocols.

Skeletal muscle comprises the most labile lean tissue reserve and is commonly sacrificed alongside adipose tissue during weight loss programmes (Forbes, 2000). To protect muscle mass, exercise regimens are generally promoted to complement dietary restriction in man (Ross *et al.*, 2000). The role of controlled exercise as both muscle-

sparing and energy-consuming components of weight loss programmes has yet to be rigorously evaluated in the horse or pony. In light of the apparent digestibility of dietary protein, intake of this nutrient may have been insufficient to meet requirements in some animals (potential 7% deficit on an actual BM basis (NRC, 2007)). Restriction of dietary protein or its metabolic combustion to offset energy shortfalls may have served to promote muscle catabolism (Phinney, 2004). Muscle catabolism was also evidenced as increased plasma creatinine concentrations in all animals, although concentrations remained within normal limits throughout the trial. This deficit highlights the importance of ensuring maintenance provision of essential nutrients, including protein, when total energy intake is restricted.

Decreasing the plane of nutrition to one third of *ad libitum* values had no significant impact on the apparent digestibility of GE or any dietary component evaluated. In the ruminant, an inverse-relationship between food intake and digestibility is well established and has been attributed to changes in the gut transit time for digesta (Warner, 1981). This classical association between plane of nutrition and apparent dietary digestibility has not been demonstrated for the horse or pony (Todd *et al.*, 1995).

Although food intake decreased to a third of *ad libitum* values with the imposition of feed restriction, time spent feeding decreased to a relatively greater extent (25% of *ad libitum*). By the end of the trial ~90% of food was being consumed within ~70 minutes of each meal presentation. Comparable increases in DMI rate and feeding times have been reported for fasted ruminants (Newman *et al.*, 1994) but this first demonstration of the effect in ponies suggests that the common practice of

housing animals for several hours to limit graze-intake may be counter-productive. Although it has previously been shown that photoperiodically-induced changes in appetite in the pony were effected by changes in meal frequency (Fuller *et al.*, 2001), imposed appetite restriction in this study (where a change in meal frequency would be limiting) elicited an increase in meal duration and consumption rate.

Decreased time spent feeding was largely offset by increased time at rest, however, these limit-fed ponies also increased time spent in non-essential activities (play), four-fold. In combination, prolonged periods without access to food, potential discomfort associated with continuous gastric acid secretion and the increased time devoted to non-essential activities, could contribute to the development of stomach ulcers and/or undesirable stereotypic behaviours (McGreevy *et al.*, 1995; Murray and Eichorn, 1996; Redbo *et al.*, 1998; NRC, 2007). Three of the five 5 ponies in the current study demonstrated trends towards increased walking or water play / polydipsia which resolved within one week of the resumption of *ad libitum* food intake. Polydipsia has previously been reported during food restriction in laboratory animals (Falk, 1969).

### **3.6 Conclusion**

This study indicated that weight loss of 1% outset BM per week can be achieved by limiting forage intake to 1% BM as DMI daily. This level of intake can only be ensured when animals are denied access to alternative forages such as straw bedding. Whereas BCS was not useful as an indicator of early weight loss, belly girth and rump width may provide proxy measures where weigh-scales are unavailable. Data suggested that this rate of weight loss might rapidly decrease hyperinsulinaemia

without compromise to health or the development of permanent undesirable behaviours. Further work is required to develop a practical nutritional protocol that could be used under field conditions. Food fibre lengths and meal frequencies could potentially be increased to prolong prehension and mastication times and reduce inter-meal intervals to limit the behavioural changes associated with periods of food deprivation (NRC, 2007). The imposition of controlled exercise and increased dietary protein provision also warrant further study to respectively promote fat mobilisation and spare lean tissue degradation.

### 3.7 References

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# **Chapter 4**

## **Assessment of body fat in the pony: I. Relationships between the anatomical distribution of adipose tissue, body composition and body condition**

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## 4.1 Summary

Evaluation of equine body fat content is important for nutritional and clinical purposes. However, our understanding of total body fat and its regional distribution in the body is sparse. Currently, body fat evaluation relies on the subjective assessment of body condition score (BCS), which has never been validated against ‘gold standard’ chemical analysis or dissection measures in ponies. We aimed to define the relationships between subjective (BCS), objective (morphometric) indices of body fat and ‘gold standard’ measures of actual body composition. Our hypothesis was that BCS and morphometry offer valid, non-invasive methods for determination of body fat in *Equidae*.

Seven mature ( $13 \pm 3$ yr,  $212 \pm 14$ kg, BCS 1.25 to 7/9), Welsh Mountain pony mares, destined for euthanasia (for non-research purposes), were used. For all ponies, body mass (BM), BCS and various morphometric measures were recorded. Following euthanasia, all ponies were systematically dissected. Discrete white adipose tissue (WAT) depots were independently described. Gross, body chemical composition was determined by proximate analyses.

Total somatic soft tissues increased linearly ( $R^2 = 1.00$ ), whereas body WAT content (1-26% Live BM) increased exponentially ( $R^2 = 0.96$ ), with BCS. WAT was equally distributed between internal and external sites in all animals irrespective of BCS. Nuchal fat was a poor predictor of total WAT ( $R^2 = 0.66$ ). Peri-orbital WAT did not alter with BCS ( $R^2 = 0.01$ ). Heart girth:withers height and ultrasonic retroperitoneal fat depth were closely associated with total, chemically-extracted lipid which comprised 1-29% Live BM ( $R^2 = 0.91$  and  $0.88$ , respectively).

The exponential relationship between BCS and total body WAT/lipid suggests that BCS is unlikely to be a sensitive index of body fat for animals in moderate-obese states. That external WAT was directly matched by internal WAT in animals of stable body condition has important implications for health and performance. Morphometric measures (body girths and retroperitoneal fat depths) may be useful to augment subjective BCS systems.

## 4.2 Introduction

Growth of the new discipline of adipobiology has been fuelled by the epidemic of human and companion animal obesity in Western civilisations (German, 2006; Sillence *et al.*, 2006; Wyse *et al.*, 2008). White adipose tissue (WAT), traditionally considered to be a passive but important contributor to energy homeostasis, is now understood to be an active secretory tissue with widespread paracrine and endocrine effects throughout the body which can impact on health (Bastard *et al.*, 2006; Vick *et al.*, 2007). Obesity has been identified as an important risk factor for morbidity and mortality in domestic horses and ponies (Sillence *et al.*, 2006; Geor, 2008). Given the importance of adipose tissue to equine health, our understanding of basic fat biology, including its contribution to body composition and its anatomical distribution, remains largely un-described (Geor, 2008; Argo, 2009).

Several authors have attempted to objectively quantify the body fat content of living horses using morphometric data, ultrasound measurement of superficial fat or bio-electrical impedance analysis (Westervelt *et al.*, 1976; Kane *et al.*, 1987; Kearns *et al.*, 2002a; Donaldson *et al.*, 2004; Frank *et al.*, 2006; Van der Aa Kuhle *et al.*, 2008; Dugdale *et al.*, 2010 and In Press). However, validation of these indirect methodologies against concurrent ‘gold standard’ carcass dissection or chemical composition analysis has been sparse, with only two studies reporting the associations between regional (rump or tailhead) subcutaneous fat depths and chemically-extracted body lipid (Westervelt *et al.*, 1976; Kane *et al.*, 1987). Inconsistencies between the actual anatomical locations for ultrasound evaluations and the physiological status of animals used, highlights the importance of defining breed-, age-, physiological status- and sex-specific, precise anatomical measurement sites (Westervelt *et al.*, 1976; Kane

*et al.*, 1987; Gentry *et al.*, 2004). Further, between-breed validation of all these indirect approaches, to confirm method associations with actual body fat content, is lacking.

Currently, the assessment of equine ‘body fatness,’ for management and clinical applications, has focused on the subjective appraisal of body condition score (BCS, Burkholder, 2000). However, unlike the successful BCS systems used in agricultural species, current equine BCS methods depend mainly on un-tested, indirect indices of total body fat mass (Henneke *et al.*, 1983; Wright and Russel, 1984). Given the current increase in the prevalence of obesity among domestic horses and ponies, a BCS system capable of quantifying body fat content is required (Thatcher *et al.*, 2007; Wyse *et al.*, 2008).

Published data documenting the gross anatomical or chemical composition of the mature equine body is scarce and confounded by differences in methodologies (Robb *et al.*, 1972; Webb and Weaver, 1979; Martin-Rosset *et al.*, 2008). Findings are limited to the observation that overall body composition is similar to that of other large mammals and that adipose tissue comprises the most variable of the major body tissues (Webb and Weaver, 1979; Gunn, 1986; Lohman, 1971). A detailed understanding of actual body fat content and the impact of changes in body fat mass on overall body composition and the regional distribution of adipose reserves would underpin future advances in methods to appraise ‘body fatness’.

This study was undertaken to generate fundamental data to accurately describe differences in body composition associated with overall differences in ‘body fatness’,

subjectively appraised as BCS and objectively evaluated by morphometric measures. A relatively homogeneous group of mature animals was used to optimize the evaluation of body fat-associated change and to minimise differences in body composition related to breed, sex or age in this instance. To facilitate between-study comparisons with future investigations, it was considered important to detail the exact procedures used to determine both the gross anatomical and chemical body composition data presented.

### 4.3 Materials and Methods

#### *Animals and husbandry*

Seven mature ( $13 \pm 3$ yr [range 6 to 20yr],  $212 \pm 14$ kg), Welsh Mountain pony mares destined for euthanasia (for non-research reasons) were used. Owner election for euthanasia had been prompted by various chronic conditions in their animals which had proved refractory to treatment (dental disease [P1 & P2], sinusitis [P3], bilateral blindness secondary to uveitis [P4], non-healing facial fistula following dental extraction [P5], infertility [P6], epilepsy [P7]). Five of the seven ponies had been fed an identical forage-based diet for 10 days prior to euthanasia; the remaining two were at pasture. Four ponies (P1 to P4) were presented during winter (February to April) and three (P5 to P7) were studied in summer (July to August).

On the day prior to euthanasia (09:00h to 10:00h), each pony was weighed ( $\pm 1$ kg, Lightweight Intermediate weigh scales; HorseWeigh, Llandrindod Wells UK; calibration regularly checked) and body condition scored (BCS 1 [very poor] to 9 [extremely fat]) in accordance with criteria described by Kohnke (1992, a modification of Henneke *et al.*, 1983) and Carroll and Huntingdon (0 [very poor] to 5 [very fat], 1988). Unless otherwise stated BCS data are reported for only the most commonly used, modified Henneke system (Suagee *et al.*, 2008). Heart and belly girths and mid-neck circumference were measured (We-Bo Animal Measure, Danish Agricultural Association) and the depths of four superficially accessible fat deposits were recorded to the nearest 0.01mm by transcutaneous ultrasonography with a variable frequency (5.5, 7 or 8MHz) linear array probe (Merlin Ultrasound scanner Type 1101; BK Medical, Herlev, Denmark), as previously described (Dugdale *et al.*, 2010; In Press).



### ***Gross dissection***

Body mass (Live BM) was recorded prior to euthanasia which was by intracranial free-bullet ( $n = 6$ ) or intravenous barbiturate overdose ( $n = 1$ ). Exsanguination was conducted immediately post-mortem and blood was collected, weighed ( $\pm 10$ g; Weigh-Tronix, Capacity 25 x 0.01kg; Avery Weigh-Tronix Ltd., West Bromwich, West Midlands, UK) and duplicate samples ( $\sim 500$ g) stored ( $-20^{\circ}\text{C}$ ) in sealed plastic containers pending chemical analyses.

All cadavers were systematically dissected (detailed description; Supplementary Information 1.1) to yield eight discrete tissue categories: collected blood; hide (including pelage); central nervous system (CNS: brain and spinal cord); right side carcass (neck, body wall and upper limbs) bones; right half head and lower limb bones (including hoof capsules); right side carcass skeletally-associated (somatic) soft tissues (white adipose tissue [WAT], skeletal muscle and other); right half head and lower limb soft tissues (WAT and other); viscera (empty) with their associated WAT. White adipose tissue (excluding intra-muscular WAT) weights from the three soft tissue categories were recorded separately from the remaining tissues in those categories. The difference between the pre-euthanasia live weight and the recovered weight (sum of the eight tissue categories plus: gastrointestinal contents, urine, peritoneal fluid, skinned left body [left carcass, head and lower fore- and hind-limbs] bones and soft tissues) was assumed to be water lost and/or gained by evaporation and/or condensation during dissection. Samples ( $\sim 1$ kg) of minced/ground tissue from each category were stored ( $-20^{\circ}\text{C}$ ) pending chemical (proximate) analyses. A sample ( $\sim 500$ g) of thoroughly mixed total digesta was also stored ( $-20^{\circ}\text{C}$ ) for later determination of dry matter (DM) content by oven-drying.

Samples (~5g fresh) of muscle (middle gluteal, semimembranosus, diaphragm and cardiac), of viscera (liver and kidney) and of various white adipose tissues (nuchal, lateral withers, tailhead, retroperitoneal, intra-pelvic, omental, mesenteric, peri-renal, pericardial and epicardial) were taken from the left half carcass (after its weight was determined) for evaluation of DM (lyophilisation) and subsequent evaluation of gross energy (GE) content (isothermal bomb calorimetry; E2K Combustion Calorimeter; Digital Data Systems (Pty), Ltd., Northcliff, South Africa; Distributed by Sartec, Ltd., Tenterden, Kent, UK).

### ***Final preparation of tissues and chemical analyses***

Samples from each tissue category were variously minced or ground and the minced/ground samples stored (-20°C) pending proximate analysis (Supplementary Information 1.1). For proximate analysis, frozen skin samples (Supplementary Information Table 1.1) were diced (~1mm square) and blood, CNS and all minced soft tissue samples were homogenised separately for each pony (Moulinex Moulinette S; Group SEB, Ecully, France). Bone samples were ground to <1mm particles (Tecator grinding mill; Tecator AB, Perstorp, Sweden) and mixed thoroughly.

Standard, proximate analytical techniques (AOAC International, 2000) were applied to each sample homogenate in triplicate (DM, lyophilisation; GE, isothermal bomb calorimetry) or in duplicate (lipid extraction, Soxhlet petroleum ether extraction before and after acid hydrolysis; nitrogen, Kjeldahl titration; ash, muffle furnace) sub-samples of the homogenates (Supplementary Information 1.1). Crude protein (CP) was calculated as nitrogen (N, g or %) x 6.25. Duplicate samples of gastrointestinal contents (digesta, ~20g) were oven-dried (75°C) to constant mass to determine DM

content. The mean values derived following replicate analyses were used in each case.

Data describing the chemical composition and total mass of each of the eight tissue categories enabled the ‘chemical reconstruction’ of each pony in terms of overall water, DM, ash, CP, neutral lipid, total lipid, and GE contents.

### ***Data analyses***

All data were initially entered into Excel spreadsheets (Microsoft Office Professional Edition 2003; Microsoft Corp., Washington, U.S.A.) and statistical analyses were performed using Excel, Minitab version 15.1.0 (Minitab Inc., Pennsylvania, U.S.A.) and STATA 10 (Stata/IC 10.1; Stata Corp., Texas, U.S.A.). Normality of all data sets for body mass, body water, ash, CP, body lipid (total and neutral) and dissected WAT (individual depots and total WAT), whether expressed as actual weights or as percentages of live or recovered empty body mass, was confirmed by both visual assessment of their frequency distributions and by Anderson-Darling normality tests. F tests were used to check for equal variance of data prior to least squares linear regression analyses. In addition, scatterplots of the data were performed to ensure that data distributions were suitable for application of linear regression; where scatterplots revealed non-linear data distribution, alternative line fits were explored in Excel. For linear regression, the outcome variables tested were: proximate analysis-derived results for body lipid, dissection-derived results for body WAT content and body condition score. Predictor variables included: body condition score, ultrasonic measures of subcutaneous and retroperitoneal fat depth, neck and body girths and all cadaver-derived measures (gut contents, bone mass,

individual organ mass, GE content, dissected depot WAT). Following linear regression, normality of the distribution of residuals was confirmed using the Anderson-Darling test. Coefficients of determination ( $R^2$ ) are reported for the results of linear regression analyses. Statistical significance was assumed if  $P < 0.05$ . Summary statistics, unless otherwise stated, are reported as mean  $\pm$  standard error.

#### 4.4 Results

By using mature animals of a single breed and sex, between-animal differences in the scale of the underlying skeletal framework, most readily evaluated as withers height ( $1.14 \pm 0.02\text{m}$ , Table 4.1a) and total skeletal mass ( $25.0 \pm 1.3\text{ kg}$ , Table 4.1b), were minimized. Changes in these markers of skeletal dimension were independent of changes in BCS (Table 4.1). Body mass varied widely across the group and increased exponentially with increasing BCS ( $R^2 = 0.64$ ). When the individual weights for all the components of each pony, following euthanasia and gross segregation of the cadaver into digesta and the major tissue categories (Table 4.1b), were re-combined, recovered body mass (recBM = recovered empty body mass [recEBM] + digesta) was strongly associated with Live BM ( $R^2 = 0.98$ ,  $P < 0.001$ , Table 4.1a). Mass deficits or excesses in recBM were minimal ( $\sim 2\%$  of Live BM) and were attributed to water lost or gained by evaporation or condensation. Net water loss during dissection tended to increase with decreasing BCS and body WAT content (Table 4.1a & b). Although the water content of digesta was almost constant between animals ( $89.5 \pm 0.9\%$ , range 86 to 92%), total digesta mass comprised  $11 \pm 2\%$  (range 7 to 20%) of Live BM and decreased logarithmically with increasing BCS ( $R^2 = 0.91$ , Table 4.1a).

**Table 4.1:** Summary data for the seven Welsh Mountain pony mares ranked in order of increasing body condition scores. **a)** age, withers height and live body mass (LiveBM) immediately prior to euthanasia. The recovered digesta mass and the recovered empty body mass (recEBM) accounted for following the summation of dissected components are presented. The percentage of LiveBM unaccounted for following dissection (and assumed to be predominantly water loss / gain) is indicated in the final column. **b)** The relative masses of each major tissue category segregated at the time of dissection are presented as percentages of recEBM for each animal. Soft tissues indicate the relative proportion of white adipose tissue (WAT), muscle and connective tissues associated with the skeleton and body wall and exclude the viscera and their associated adipose tissues (vWAT), hide and pelage, collected blood and central nervous system (CNS) tissues which are separately listed. Mean and standard errors (se) are presented for each variable.

a)

Pony	BCS (1-9)	Age (yr)	Withers height (m)	Live BM (kg)	Digesta (kg)	RecEBM (kg)	RecEBM + digesta (% Live BM)	Unaccounted mass (% Live BM)
<b>1</b>	1.25	12	1.15	173.0	34.00	129.74	94.65	5.35
<b>2</b>	2.5	9	1.15	159.0	20.00	141.83	101.78	-1.78
<b>3</b>	4.08	11	1.17	214.0	24.46	179.49	95.30	4.70
<b>4</b>	4.25	17	1.16	238.0	21.00	212.01	97.90	2.10
<b>5</b>	5.9	20	1.08	211.0	21.00	191.48	100.70	-0.70
<b>6</b>	6.8	6	1.07	220.0	16.00	198.26	97.39	2.61
<b>7</b>	7	16	1.17	270.0	21.50	243.21	98.04	1.96
<b>mean</b>		<b>13.00</b>	<b>1.14</b>	<b>212.14</b>	<b>22.57</b>	<b>185.15</b>	<b>97.97</b>	<b>2.03</b>
se		1.85	0.02	14.17	2.13	14.87	0.98	0.98

b)

Pony	BCS (1-9)	Major tissues as a percentage of recovered empty BM							
		Musculoskeletal system				Viscera and vWAT	Hide and pelage	Collected blood	CNS
		Bone & cartilage	Soft Tissue						
			Total	WAT	Non-WAT				
1	1.25	19.74	49.49	1.18	48.31	15.22	7.98	7.09	0.48
2	2.5	18.90	54.01	5.09	48.91	13.01	7.72	5.92	0.44
3	4.08	15.02	57.87	12.38	45.49	13.21	6.82	6.70	0.38
4	4.25	13.92	58.77	19.37	39.40	13.00	8.16	5.84	0.30
5	5.9	11.07	63.48	30.86	32.61	14.94	5.95	4.21	0.34
6	6.8	9.96	65.70	47.09	18.61	14.08	4.50	5.40	0.36
7	7	10.42	65.95	45.12	20.83	12.23	5.38	5.75	0.27
mean		14.15	59.32	23.01	36.31	13.67	6.65	5.85	0.37
se		1.51	2.34	6.99	4.79	0.42	0.53	0.35	0.03

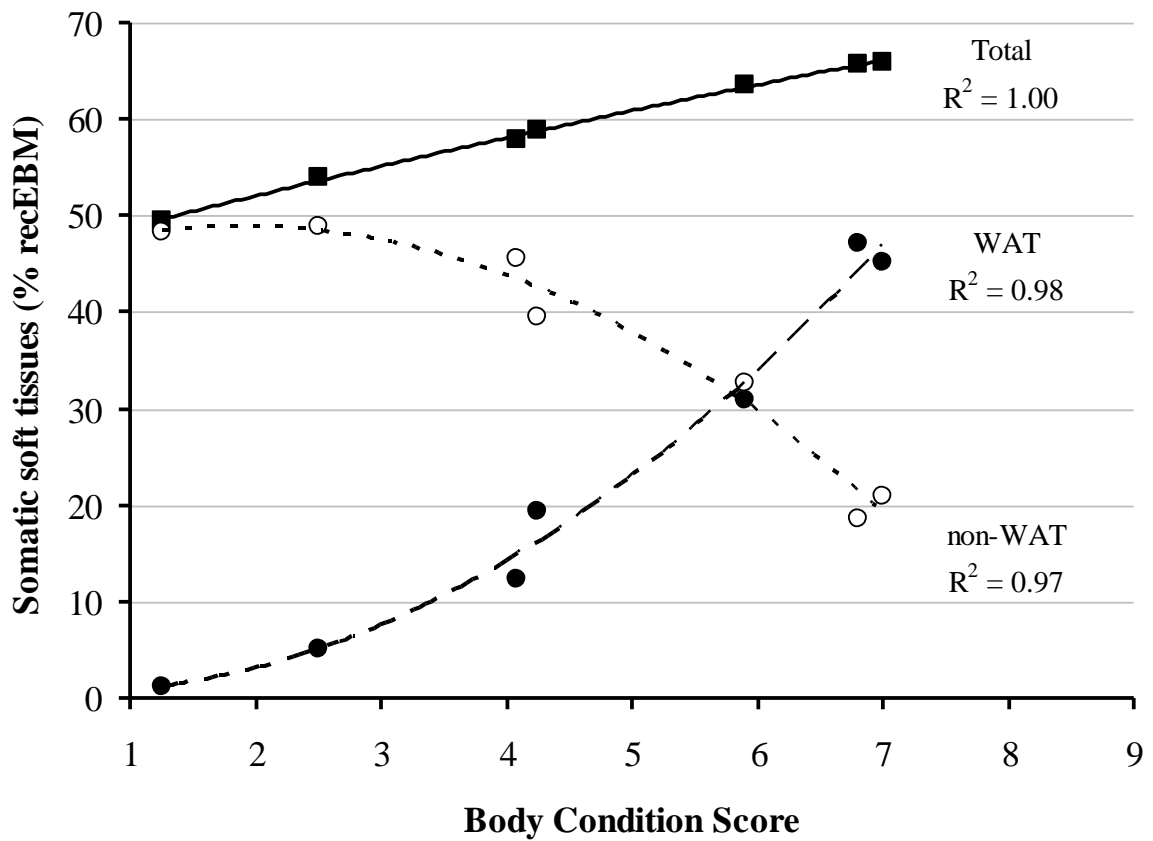
### ***Gross anatomical evaluation***

Skeletally-associated (somatic) soft tissue was both the largest component of each cadaver and the most variable between animals when considered as a proportion of recEBM (Figure 4.1, Table 4.1b). Subjective measures of BCS, almost perfectly, linearly, described ( $R^2 = 1.00$ ,  $P < 0.001$ ) total somatic soft tissue (WAT + non-WAT) contributions to recEBM (Figure 4.1, Table 4.1b). However, as BCS increased, the relative contribution of WAT and non-WAT (skeletal muscle and connective tissues) within the somatic soft tissues, changed in a reciprocally curvilinear manner (Figure 4.1, Table 4.1b).

The absolute mass of collected blood ( $10.7 \pm 0.8\text{kg}$ ) and CNS tissues ( $655 \pm 12\text{g}$ ) was relatively constant between ponies. Although hide (including pelage) mass varied little between ponies ( $12.0 \pm 1.0\text{kg}$ ), a seasonal effect was apparent when hide was expressed as a percentage of recEBM ( $P = 0.03$ ; winter,  $6.5 \pm 0.4\text{kg}$ ; summer,  $4.8 \pm 0.4\text{kg}$ , Table 4.1b).

When the relatively small proportion of total WAT associated with the head and lower limbs ( $2.3 \pm 0.8\%$ ) was excluded, WAT was essentially equally-distributed between the internal and external carcass sites in all animals irrespective of BCS (Table 4.2). Although anatomically-regional WAT depots contributed variously to the total WAT mass in individual ponies, the most profound changes associated with increased total body WAT content were attributed to increased mass in three principal regional deposits; intra-abdominal belly wall-associated (retroperitoneal), subcutaneous and inter-muscular (Table 4.2).





**Figure 4.1:** The recovered mass of total somatic soft tissues (skeletally-associated, ■) and the components of this soft tissue mass which were comprised of white adipose tissue (WAT, ●) and other, non-WAT (largely skeletal muscle) tissues (○), for the seven, Welsh Mountain pony mares are illustrated against their individual, *ante-mortem* body condition scores. Coefficients of determination ( $R^2$ ) are presented.

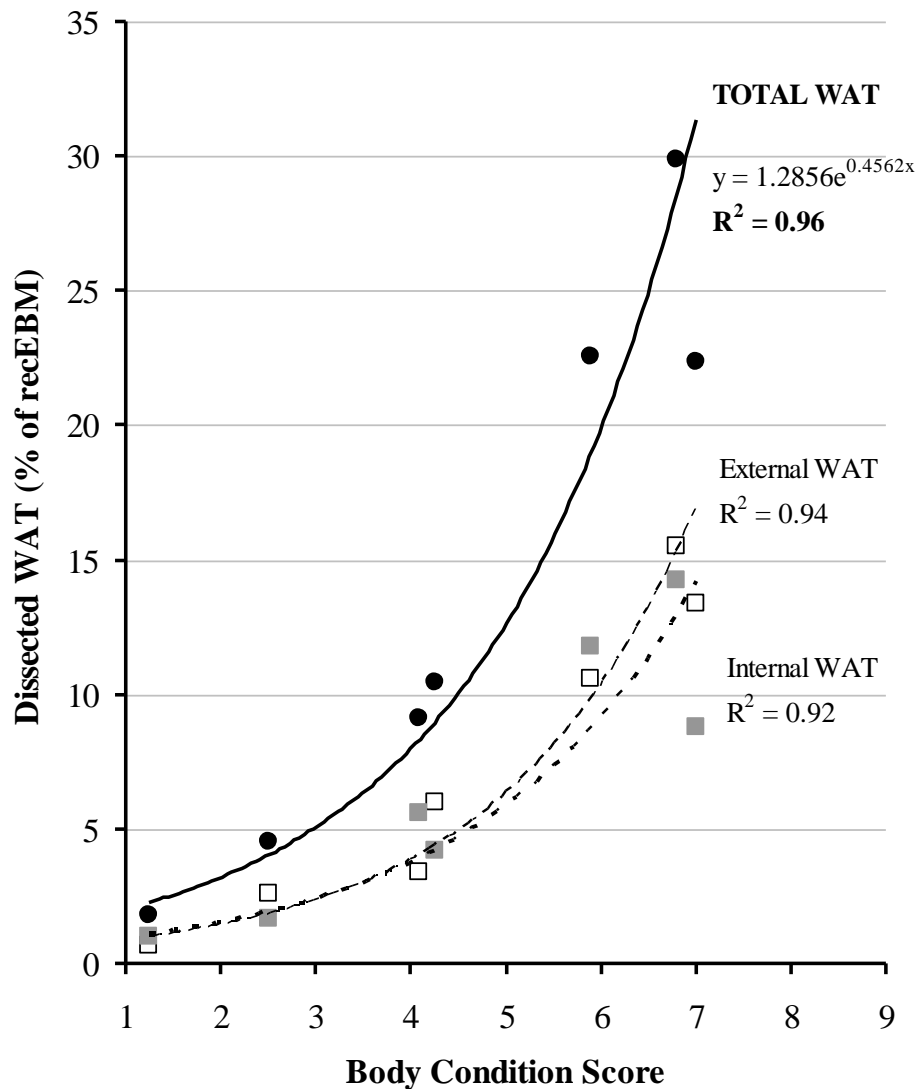
**Table 4.2:** Data from the seven Welsh Mountain pony mares are presented in order of increasing body condition score. Regional white adipose tissue (WAT) depots from four main sites (a to d) are presented as percentages of recovered empty body mass.

<b>Pony BCS</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>Mean</b>	<b>se</b>
<b>a) Internal carcass (body wall-associated)</b>									
Intra-thoracic	0.06	0.23	0.20	0.27	0.43	0.48	0.48	0.31	0.06
Intra-abdominal	0.00	0.36	3.07	1.15	4.12	7.38	3.98	2.87	0.98
Intra-pelvic	0.05	0.15	0.09	1.50	0.82	0.23	0.51	0.48	0.20
<b>Total (a)</b>	<b>0.11</b>	<b>0.74</b>	<b>3.36</b>	<b>2.92</b>	<b>5.37</b>	<b>8.09</b>	<b>4.97</b>	<b>3.65</b>	<b>1.05</b>
<b>b) Internal carcass (organ-associated)</b>									
Omental	0.05	0.06	0.28	0.05	1.33	0.74	0.36	0.41	0.18
Mesenteric	0.68	0.44	1.44	0.58	4.26	4.53	2.70	2.09	0.66
Peri-renal	0.03	0.07	0.16	0.17	0.25	0.22	0.17	0.15	0.03
Peri-cardial	0.08	0.11	0.09	0.14	0.16	0.05	0.23	0.12	0.02
Epicardial	0.04	0.07	0.05	0.12	0.09	0.06	0.12	0.08	0.01
Broad ligament	0.00	0.16	0.22	0.22	0.34	0.54	0.25	0.25	0.06
<b>Total (b)</b>	<b>0.88</b>	<b>0.91</b>	<b>2.24</b>	<b>1.28</b>	<b>6.43</b>	<b>6.14</b>	<b>3.83</b>	<b>3.1</b>	<b>0.91</b>
<b>Total Internal (a+b)</b>	<b>0.99</b>	<b>1.65</b>	<b>5.6</b>	<b>4.2</b>	<b>11.8</b>	<b>14.23</b>	<b>8.8</b>	<b>6.75</b>	<b>1.9</b>
<b>c) External carcass (palpable)</b>									
Subcutaneous	0.03	0.74	1.31	0.85	2.08	5.69	4.42	2.16	0.79
Udder-associated	0.01	0.01	0.11	0.00	0.11	0.37	0.34	0.14	0.06
Nuchal	0.01	0.81	0.4	0.32	0.32	1.55	1.15	0.65	0.21
Inter-muscular	0.64	1.06	1.58	4.85	8.08	7.88	7.47	4.51	1.27
<b>Total External (c)</b>	<b>0.69</b>	<b>2.62</b>	<b>3.4</b>	<b>6.02</b>	<b>10.59</b>	<b>15.49</b>	<b>13.38</b>	<b>7.46</b>	<b>2.16</b>
<b>d) Head / lower limb</b>									
Peri-orbital	0.07	0.09	0.05	0.06	0.06	0.04	0.05	0.06	0.01
Other	0.02	0.16	0.09	0.15	0.09	0.12	0.15	0.11	0.02
<b>Total head (d)</b>	<b>0.09</b>	<b>0.25</b>	<b>0.14</b>	<b>0.21</b>	<b>0.15</b>	<b>0.16</b>	<b>0.20</b>	<b>0.17</b>	<b>0.02</b>
<b>GRAND TOTAL (a+b+c+d)</b>	<b>1.77</b>	<b>4.52</b>	<b>9.14</b>	<b>10.43</b>	<b>22.54</b>	<b>29.88</b>	<b>22.38</b>	<b>14.38</b>	<b>3.99</b>

Successive, linearly-ordinal increments in BCS were associated with an exponential increase ( $R^2 = 0.96$ ) in the proportion of WAT within the recEBM (Figure 4.2). (Similarly, an exponential relationship was present between WAT expressed as a percentage of LiveBM and BCS,  $R^2 = 0.95$ , data not shown.) A similar exponential increment in total body WAT (1-26% Live BM) was observed when changes in body condition were independently appraised using the 0 to 5 system of Carroll and Huntingdon (1988,  $R^2 = 0.90$ ; data not presented). Internal and external WAT, inclusive of their component depots, contributed equally to BCS-associated changes in total WAT (Figure 4.2; Table 4.2). Further, the two major categories which comprised internal WAT, body wall-associated and organ-associated, also appeared to contribute equally to changes in total internal WAT. The animal for which the lowest BCS was recorded (P1) was the exception to this general trend. For this individual, mesenteric WAT, the largest organ-associated depot in all ponies, comprised 68% of total internal WAT, in the absence of any dissectable retroperitoneal WAT (Table 4.2; Supplementary Information Table 1.2). In addition, all dissectable WAT in this animal was of a gelatinous nature. This was particularly notable between the dorsal spinous processes of the vertebrae.

As BCS increased, increasing quantities of inter-muscular WAT were evident between the major muscle bellies (Table 4.2). Inter-muscular WAT recorded for the animal in poorest BCS (P1) consisted mainly of peri-articular WAT and WAT associated with neurovascular and lymphoid tissues. Intra-muscular WAT was not amenable to dissection and remained un-quantified but was grossly visible in only one animal (P7). In this individual, intra-muscular WAT was apparent as ‘marbling’ between individual muscle fascicles throughout *quadriceps femoris* alone (Figure

4.3). Although digital cushions were not removed from hoof capsules, the remaining lower limb WAT was negligible (<5g) in all ponies, was independent of BCS and was mainly peri-articular or associated with neurovascular and lymphatic tissue.



**Figure 4.2:** Total, white adipose tissue mass (WAT, black circles) and its distribution between the ‘external’ (subcutaneous and inter-muscular, white squares) and ‘internal’ (body wall- and organ- associated, grey squares) body compartments for the seven Welsh Mountain ponies are regressed on their respective *ante-mortem* body condition scores (BCS). The coefficients of determination ( $R^2$ ) for each data set and the exponential equation describing the overall relationship between BCS and total WAT as a percentage of recovered body mass are presented.



**Figure 4.3:** Marbling of fat between muscle fascicles of *quadriceps femoris* (pony 7).

Regression of each WAT depot on total WAT mass demonstrated strong, positive associations for most depots. Body wall-associated intra-abdominal (retroperitoneal,  $R^2 = 0.89$ ,  $P = 0.001$ ) and intra-thoracic (retropleural,  $R^2 = 0.92$ ,  $P < 0.001$ ) depots were strongly associated with total WAT mass, whereas the intra-pelvic depot was not ( $R^2 = 0.05$ ,  $P = 0.6$ ). Of the organ-associated WAT depots, mesenteric ( $R^2 = 0.88$ ,  $P = 0.002$ ), uterine broad ligament ( $R^2 = 0.83$ ,  $P = 0.004$ ) and perirenal ( $R^2 = 0.78$ ,  $P = 0.008$ ) depots demonstrated the strongest associations with total WAT mass. Conversely, omental ( $R^2 = 0.51$ ,  $P = 0.07$ ) and heart-associated WAT

(pericardial and epicardial depots,  $R^2 < 0.3$ ,  $P > 0.2$ ), were poorly associated with total WAT mass. Inter-muscular ( $R^2 = 0.91$ ,  $P = 0.001$ ) and subcutaneous ( $R^2 = 0.87$ ,  $P = 0.002$ ) depots were more strongly associated with total WAT than the other two depots of external WAT (udder-associated,  $R^2 = 0.77$ ,  $P = 0.009$ ; and nuchal,  $R^2 = 0.66$ ,  $P = 0.03$ ). Nuchal fat was grossly different, firmer and paler than other WAT deposits. Peri-orbital ( $R^2 = 0.01$ ,  $P = 0.9$ ) and other head WAT ( $R^2 = 0.3$ ;  $P = 0.2$ ) were independent of total WAT mass and BCS.

Total mass of ‘WAT-denuded’ viscera (thoracic and abdominal) increased linearly (slope  $< 0.1$ ) as WAT-free, recEBM increased ( $R^2 = 0.63$ ,  $P = 0.03$ ; Supplementary Information Table 1.3). However, total organ mass (WAT-denuded) was more usefully described as a proportion of WAT-free recEBM. On this basis, the proportion of WAT-free recEBM comprised of WAT-denuded viscera decreased linearly as BCS increased ( $R^2 = 0.84$ ,  $P = 0.004$ ) but the contribution of specific viscera to this overall trend differed. The proportions of WAT-free recEBM comprised of heart and liver were independent of changes in BCS ( $R^2 < 0.1$ ,  $P > 0.5$ ). Conversely, the proportion of WAT-free recEBM comprised of gastrointestinal ( $R^2 = 0.89$ ,  $P = 0.001$ ) and respiratory ( $R^2 = 0.67$ ,  $P = 0.03$ ) tracts, decreased markedly as BCS increased.

The major body organs, once denuded of their WAT, contributed variously to Live BM (CNS,  $0.32 \pm 0.02\%$ ; heart,  $0.62 \pm 0.02\%$ ; respiratory tract,  $0.98 \pm 0.06\%$ ; empty gastro-intestinal tract,  $5.33 \pm 0.48\%$ ; liver,  $1.20 \pm 0.11\%$ ; pancreas,  $0.11 \pm 0.01\%$ ; spleen,  $0.25 \pm 0.02\%$ ; kidneys,  $0.34 \pm 0.03\%$ ; empty urogenital tract,  $0.37 \pm 0.06\%$ ).

### ***Gross chemical evaluation***

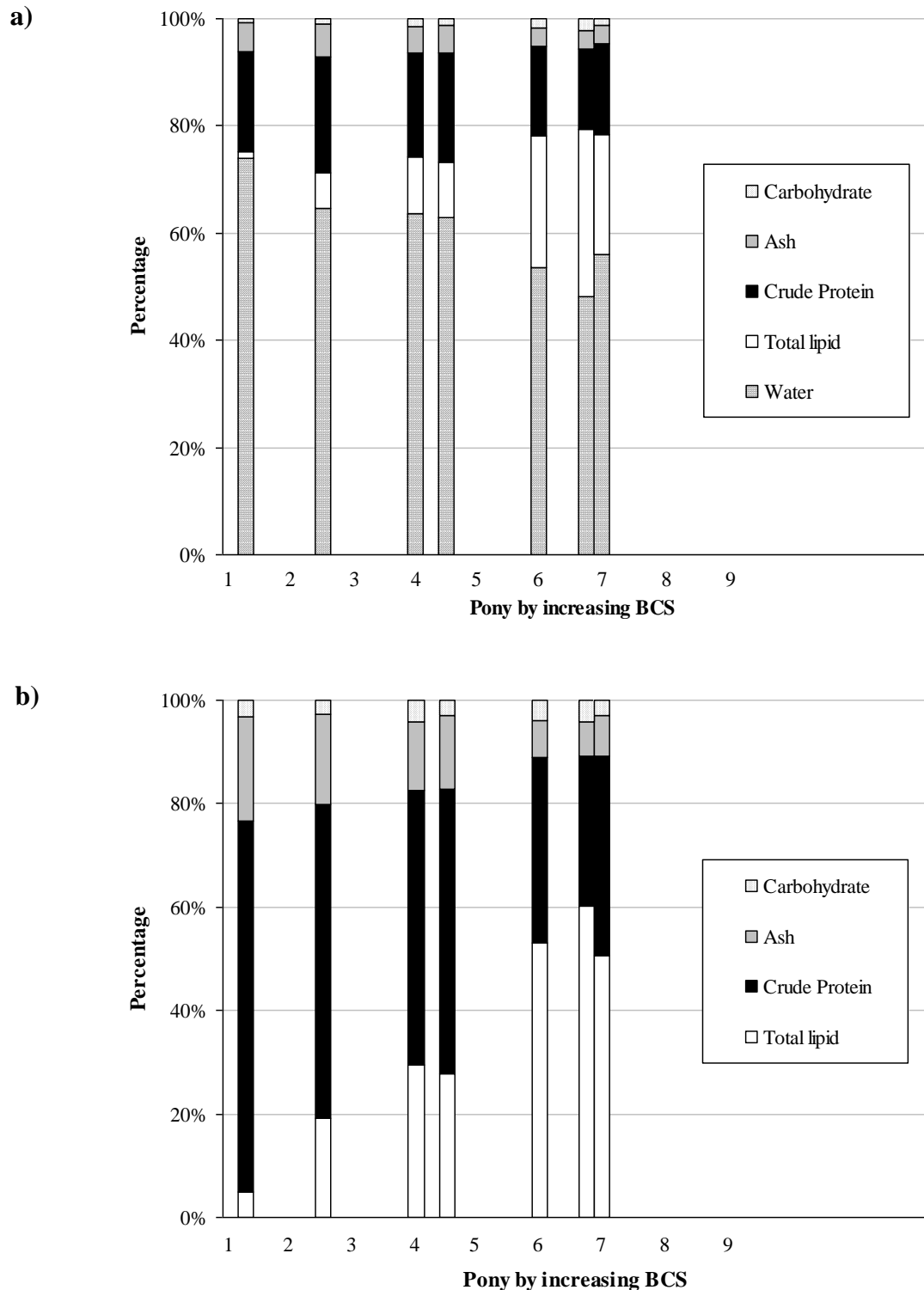
Chemical analyses of all cadaver parts allowed the gross chemical reconstitution of each pony (Figure 4.4; Supplementary Information Table 1.4). Chemically-reconstituted empty body mass over-estimated recEBM by  $2.5 \pm 1.2\%$ . Chemically-reconstituted empty body mass (Figure 4.4a) comprised: water,  $60.4 \pm 3.2\%$  (range 48.2 to 73.9%); crude protein,  $18.4 \pm 0.9\%$  (range 15.0 to 21.6%); total (neutral plus polar) lipids,  $15.3 \pm 4.1\%$  (range 1.3 to 31.1%); and ash,  $4.6 \pm 0.4\%$  (range 3.4 to 6.2%). Carbohydrates (assumed mainly glycogen) were not analysed but calculation by difference would suggest that they accounted for no more than  $1.4 \pm 0.2\%$  of chemically-reconstituted empty body mass.

Body lipid mass, whether expressed as neutral body lipid (NBL, mainly 'storage' triglycerides) or total body lipid (TBL = NBL plus 'structural', polar lipids), was strongly associated with the mass of total dissected WAT ( $R^2 = 0.99$ ,  $P < 0.001$ ). NBL comprised 0.8 to 31.1% recEBM (0.6 to 28% Live BM) and TBL comprised 1.3 to 32.0% recEBM (1 to 29% Live BM). Structural lipids (calculated by difference, TBL - NBL), comprised  $9 \pm 5\%$  (range 1.5-38.5%) of TBL and their relative contribution to TBL was inversely associated with BCS. However, structural lipids comprised a relatively constant  $0.6 \pm 0.1\%$  of recEBM, regardless of BCS.

Body water comprised  $63.3 \pm 3.2\%$  (range 51.4 to 77.1%) of Live BM,  $62.0 \pm 3.7\%$  (range 49.6 to 79.2%) of recEBM and varied inversely with the percentage of total and neutral body lipid ( $R^2 = 0.94$ ,  $P < 0.001$ ; Figure 4.4a; Supplementary Information Table 1.4). This relationship was also evident at tissue level. The water content of four different muscles ( $R^2 = 0.81$ ,  $P = 0.015$ ) and up to ten different

adipose tissues ( $R^2 = 0.80$ ,  $P = 0.016$ ) varied inversely with BCS (Supplementary Information Table 1.5). Total body lipid constituted  $13.9 \pm 3.8\%$  (range 1.0 to 28.8%) of Live BM ( $15.6 \pm 4.2\%$  of recEBM; range 1.3 to 32.0%), of which neutral lipids were the majority component ( $13.4 \pm 3.8\%$  [range 0.6 to 28.0%] of Live BM;  $15.0 \pm 4.1\%$  recEBM [range 0.8 to 31.1%]). Crude protein comprised  $16.32 \pm 0.73\%$  Live BM ( $18.84 \pm 0.89\%$  recEBM), carbohydrate  $1.23 \pm 0.17\%$  Live BM ( $1.41 \pm 0.8\%$  recEBM) and ash  $4.03 \pm 0.34\%$  Live BM ( $4.67 \pm 0.43\%$  recEBM). As body lipid content increased, the non-lipid components of body dry matter (protein and ash) decreased (Figure 4.4b; Supplementary Information Table 1.4).





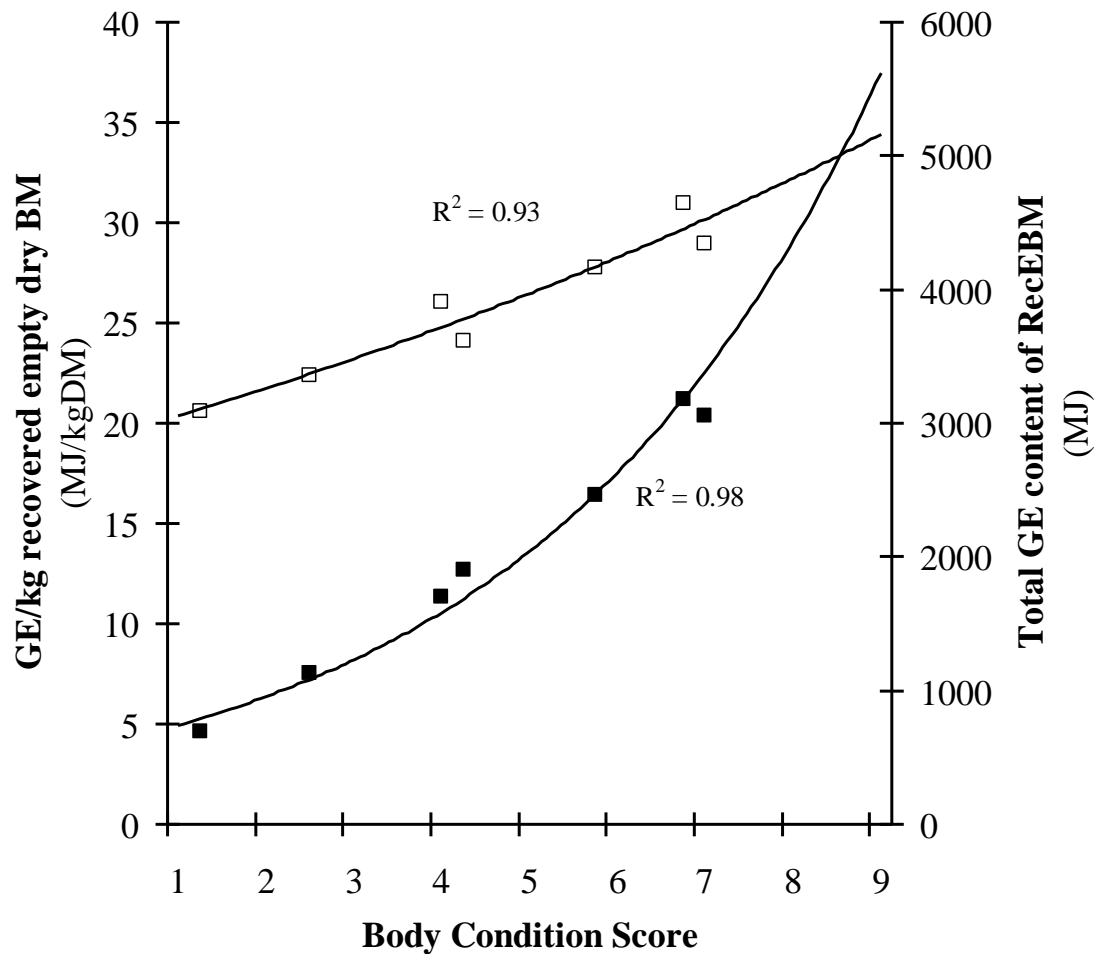
**Figure 4.4:** Gross chemical body compositions (water, crude protein, total [neutral and polar] lipid, ash and by difference, carbohydrate) as determined by proximate analyses, are presented as percentages of the total empty body in composite histogram bars for each of the seven animals. Data for body composition **a)** as fresh tissues and **b)** as dry matter are ranked for each animal against their *ante-mortem* body condition scores.

Correlation and regression analyses of all analytes for each of the 8 dissected tissue categories indicated that the chemical composition of CNS and hide were conserved in the face of changing BCS (Supplementary Information Table 1.4). For all other tissue categories, water and lipid contents were inversely associated. This was exemplified by changes in the chemical composition of somatic soft tissues and viscera (Supplementary Information Table 1.4). Blood lipid contents were greatest for two animals, one of low BCS (P2) and one with the highest BCS (P7).

Total body GE content increased exponentially with BCS (Figure 4.5). For ponies of moderate and obese BCS, the GE content of WAT DM was similar, both between animals and between anatomically distinct WAT deposits ( $42.0 \pm 0.2 \text{ MJ/kgDM}$ ), although there was a trend for nuchal WAT to contain least energy while retroperitoneal WAT was the most calorific reserve (Supplementary Information Table 1.6). Insufficient WAT was harvested from lean animals (P1 & P2) to allow replicate evaluations of adipose tissue-specific GE for each lean individual, such that only a single determination was possible which suggested a lower GE content than WAT from moderate and obese animals ( $27.6 \text{ MJ/kgDM}$ ).

Evaluation of morphometric data indicated that belly girth was more strongly correlated with total chemically-extracted lipid ( $R^2 = 0.73$ ,  $P = 0.014$ ) than mid-neck circumference ( $R^2 = 0.64$ ,  $P = 0.02$ ) and heart girth ( $R^2 = 0.56$ ,  $P = 0.54$ ). When normalised for withers height, heart girth was more strongly associated with total chemically-extracted lipid ( $R^2 = 0.91$ ,  $P = 0.001$ ) than belly girth ( $R^2 = 0.82$ ,  $P = 0.005$ ) and mid-neck circumference ( $R^2 = 0.75$ ,  $P = 0.01$ ). In addition, retroperitoneal fat depth, measured by transcutaneous ultrasonography, demonstrated the strongest

correlation with total chemically-extracted body lipid ( $R^2 = 0.88$ ,  $P = 0.002$ ). Tailhead ( $R^2 = 0.51$ ,  $P > 0.05$ ), 12<sup>th</sup> rib-eye ( $R^2 = 0.19$ ,  $P = 0.3$ ) and gluteal ( $R^2 = 0.15$ ,  $P = 0.4$ ) sites were not useful predictors of body lipid content in these seven pony mares.



**Figure 4.5:** Body condition score for each of the seven Welsh Mountain pony mares regressed on total gross energy (GE) content of the recovered empty body mass (black squares) and the GE content of each kg of dry matter (white squares). Coefficients of determination ( $R^2$ ) are presented.

## 4.5 Discussion

In companion animal management, BCS has become accepted as a useful monitor of body ‘fatness’ (La Flamme, 1997a & b). However, in this relatively homogeneous group of animals, while BCS offered an almost perfect index of total somatic (skeletally-associated) soft tissues, it was markedly less useful in predicting total body fat. This finding serves as an important reminder that BCS systems were originally developed by the food animal industry for the evaluation of superficial ‘flesh’ in general and were not intended for the evaluation of fat alone (Jeffries, 1961). To date, data validating the use of BCS systems against ‘gold standard’ cadaver dissection or chemical composition analysis are sparse for all species and only a small number have addressed the ability of BCS to discriminate between body fat and muscle (Russel *et al.*, 1969; Wright and Russel, 1984; Otto *et al.*, 1991; Gregory *et al.*, 1998; Martin-Rosset *et al.*, 2008).

An understanding of the non-linear relationship between current equine BCS systems and body fat content was gained by exploration of the relative contribution of WAT to the total somatic soft tissues. In the horse and other animals, quantitatively, WAT comprises the most variable of all body tissues (Lohman, 1971; Webb and Weaver, 1979). In contrast to the precise linear relationship between subjective BCS and total somatic soft tissue, total body WAT content increased exponentially with BCS irrespective of the specific BCS system used. Current data, although restricted to animals of BCS 7/9 and below, suggest that the sensitivity of BCS for the quantification of body WAT or lipid content, is decreased when BCS exceeds 5-6/9 (Dugdale *et al.*, In Press). This loss of sensitivity is associated with the increasing slope of the relationship, coincident with a region of the BCS scale where subjective

descriptors of body condition inadequately distinguish between animals for which bony anatomical landmarks are already obscured by soft tissue (Dugdale *et al.*, In Press). Similar exponential or curvilinear relationships between total body fat and BCS have been demonstrated for horses and cattle (Gregory *et al.*, 1998; Martin-Rosset *et al.*, 2008). These data suggest the need to identify more precise descriptors or objective measures of body fat in animals in moderate to obese condition to augment the precision of current BCS systems.

In this study, changes in BCS provided a valid, linear method for the determination of the highly variable, total somatic soft tissue content (2.8% of recEBM / BCS point; or 3.5% of Live BM / BCS point). For lean animals, non-WAT soft tissues (largely skeletal muscle) comprised ~40% Live BM (~50% recEBM), less than reported for racing breeds (53-57% Live BM, Kearns *et al.*, 2002b), which probably reflected differences in breed and/or fitness. In contrast to data for humans, particularly women, and unfit Standardbred mares, a reciprocal relationship was evident between WAT and non-WAT soft tissues with increased BCS, suggesting that lean tissue loss may accompany WAT deposition (Forbes, 1987; Kearns *et al.*, 2002b; Dugdale *et al.*, In Press).

The relative distribution of WAT across different anatomical regions was largely independent of BCS in these ponies. This agreed with comparable reports for cattle and sheep (Russel *et al.*, 1971; Wright and Russel, 1984). In the current study, WAT was equally distributed between ‘internal’ (body wall / organ-associated) and ‘external’ (inter-muscular / subcutaneous) deposits. Uniformity of WAT distribution between the ‘internal, covert’ and ‘external, potentially palpable’ reserves, would lend

credence to the view that the quantitative appraisal of external reserves by clear measures or descriptors (BCS) should provide a useful index of total WAT. However, animals in the current trial had been in their final BCS for a prolonged period before study, whereas animals undergoing active weight change may favour different regional deposits during fat deposition or mobilisation (Dugdale *et al.*, 2010; Dugdale *et al.*, In Press). This observation is supported by work in horses and other mammalian species which suggests that WAT accretion and depletion follows a pre-set order, ‘fat patterning’, which may be species-, breed-, age- and individual- specific (Riney, 1955; Russel *et al.*, 1971; Westervelt *et al.*, 1976; Butler-Hogg, 1984; Butler-Hogg *et al.*, 1985; Pond, 1998; Gentry *et al.*, 2004; Carter *et al.*, 2009). On this basis, the quantitative appraisal of BCS (external WAT) has the potential to misinform when used to monitor changes in total body WAT.

Evaluation of the nature and distribution of WAT between specific regional deposits in animals of different BCS reinforced the physiological distinctions between the major ‘storage’ deposits and lesser ‘structural’ and ‘functional’ WATs. These latter WAT categories are, respectively, essential for mechanical support and local protection within the body and the storage and release of triglycerides for buffering the metabolic requirements of nearby tissues (Pond, 1998). It has been suggested that ‘structural’ (e.g. peri-orbital and peri-articular) and ‘functional’ WAT reserves associated with the heart, surrounding lymph nodes and within bone marrow, may be the last to be depleted in the face of chronic starvation (Pond, 1998). At the thin end of the BCS spectrum (P1, BCS 1.25/9), all ‘functional’ WAT deposits were depleted and adipose tissue remnants demonstrated gelatinous, serous atrophic changes consistent with active mobilisation (Schoonderwoerd *et al.*, 1986). Despite this, the

relative mass of ‘structural’, peri-orbital adipose tissue had been conserved in this animal. Other ‘structural’ WAT deposits, including adipose tissue associated with the digital cushions and peri-articular fat pads were not specifically recorded. That the lipid content of bone marrow, a ‘functional’ WAT depot, was reduced (P1) when compared with that of the other animals, supported findings in ruminants in which bone marrow lipid was only depleted once chronic starvation had reduced body fat content to below ~5% (Pond, 1998; Wright and Russel, 1984; Russel *et al.*, 1971; Riney, 1955). At the higher end of the BCS spectrum, however, there may be a maximum capacity of the bone marrow to store lipid. Russel and colleagues (1971) suggested that once body fat percentage exceeded about 15% in sheep, little extra lipid could be stored in the bone marrow, which would appear in agreement with our data from these ponies.

Of special interest, whilst at the low end of the BCS spectrum, ‘structural’ peri-orbital adipose tissue was conserved (P1, BCS 1.25), at the higher end of the studied BCS range (P6 & P7, BCS 6.8 & 7.0), palpably and quantitatively enlarged deposits of WAT were associated with the udder (both animals had had foals). Evaluation of udder and/or preputial fat, considered a ‘structural depot’, may therefore offer a useful aid to BCS assessment in overweight animals (Pond, 1998).

Although the relative masses of the majority of superficially accessible fat depots, which contributed to both BCS systems, were strongly associated with total body WAT, nuchal ligament fat was the least dependable variable in the current study. This observation re-iterates the importance of appraising fat deposition for BCS estimation at several discrete anatomical sites (Burkholder, 2000). Crest fat thickness

has previously been reported as a good indicator of total empty carcass fat ( $R^2 = 0.77$ ), however these data were derived from 107 horses grouped towards the lower end of the expected range of total body fat contents ( $7.85 \pm 4.87\%$ ; range 1.2 to 18.5%), where the sensitivity of BCS descriptors for the determination of body fat would have been greatest (Znamierowska, 2005). Nuchal fat may differ from other fat deposits as it also has a functional and sexually dichotomous role, evidenced by a higher content of connective tissue, which would also account for the lower energy concentrations determined in this study and previously reported for this reserve (Korzeniowski *et al.*, 1994; Pond, 1998). The GE contents of different pony tissues were almost identical to those reported for a limited range of bovine tissues (Blaxter and Rook, 1953). Notably, the GE content of adipose tissues for ponies in higher BCS exceeded published values for cattle (41 to 43 MJ/kgDM cf. 39 MJ/kgDM, Blaxter and Rook, 1953). However, recent reports may support the hypothesis that different regional WAT depots are metabolically distinct but the physiological relevance of these differences has yet to be determined (Burns *et al.*, 2009; Suagee *et al.*, 2010).

The relative contributions of the gross tissue categories (hide, collected blood, bones etc.) to the recovered empty (digesta excluded) body of the mature pony mares were consistent with data published for horses, cattle, sheep and pigs (Webb and Weaver, 1979; Ockerman and Hansen, 2000). Similarly, central nervous system and individual visceral organ weights were in agreement with ranges published for horses (Bradley, 1896; Webb and Weaver, 1979). Seasonal differences in hide weights were most probably associated with differences in pelage mass. Of note, in this study and that of Webb and Weaver (1979), was that the equine heart (0.6% Live BM) and CNS



(0.3% Live BM) appeared to be relatively larger than those of pigs and ruminants (0.3-0.5% and 0.1-0.15% Live BM, respectively; Ockerman and Hansen, 2000).

The observation that gut fill varied inversely with BCS in these ponies for which forage was available *ad libitum* reinforces an important issue. It has previously been demonstrated that both appetite and maintenance energy requirements are decreased in obese ponies and cattle (Bines *et al.*, 1969; Dugdale *et al.*, In Press). For cattle, it has been suggested that decreased appetite is secondary to physical constraints on gut capacity as a result of increased intra-abdominal fat (Bines *et al.*, 1969). Conversely, it could be argued that decreased appetites are secondary to decreased metabolic requirements in obese subjects (Woods and D'Alessio, 2008). However, total gastro-intestinal tract mass also decreased with increasing BCS. Atrophy of gut tissues has previously been reported for other herbivorous mammals when voluntary food intake decreased (Gross *et al.*, 1985; Rhind *et al.*, 2002). Further study is required to unravel the relationships between obesity, appetite and gastro-intestinal function. Despite the decreased gut fill of obese animals, belly girth retained its strong association with BCS, a relationship which had been demonstrated in previous studies (Dugdale *et al.*, In Press). Retroperitoneal fat depth and body girths provided reasonably objective measures of whole body adiposity in this study and may offer practical tools for monitoring body fat in ponies in a manner comparable to that of waist circumference measures in humans (Lean *et al.*, 1996). Such simple measures would be easily obtainable in the clinical setting and may provide useful markers, mainly for monitoring changes in body condition, since one-off measures are unlikely to be consistently related to body fat content across all breeds and sexes (Westervelt *et al.*, 1976; Reavell, 1999). The sheer bulk of intra-thoracic WAT in the

overweight ponies was alarming and may have serious implications for respiratory function for health and athletic performance, especially since none was scored higher than BCS 7/9 (Lazarus *et al.*, 1998; Mansel and Clutton, 2008).

In gross chemical terms, the composition of the empty pony body was comparable (water, ~ 62%; CP, ~19%; lipid, ~16%; ash, ~5%) to that of beef and dairy cattle and horses (Reid *et al.*, 1955; Kane *et al.*, 1987). Carbohydrates, mainly muscle glycogen, have been reported to comprise less than 0.5% of the human and bovine body (Reid *et al.*, 1955; Sheng and Huggins, 1979). However, current estimates of carbohydrate content exceeded those for cattle, which would agree with previous reports which indicate that the glycogen content of flight-adapted horse muscle is greater than that of cattle (Lindholm and Piehl, 1974; Immonen *et al.*, 2000). The actual nitrogen content of equine tissue protein has not been determined. Application of the generic 6.25 conversion factor used to estimate CP content from tissue nitrogen concentrations may have contributed to the 2.5% over-estimation when body mass was re-calculated as the sum of its chemical components. One cattle study proposed an alternative correction factor (5.8) which more correctly accounted for the nitrogen content of bovine tissue proteins (Odwongo *et al.*, 1984). The nitrogen content of equine-specific tissue proteins has yet to be determined.

The chemical compositions of hide, bone and, to all intents and purposes, blood, were consistent between ponies and similar to values reported for cattle (Ferrell and Jenkins, 1984; Nour and Thonney, 1987; Ockerman and Hansen, 2000).

The reciprocal relationship between body water and body lipid content may have accounted for the greater dissection-associated water losses recorded for lean animals (Siri, 1956). The range of total lipid content of these ponies was greater (up to 29% Live BM or 32% recEBM) than has been suggested but previous reports tended to use leaner animals (6.6-18.9% of empty BM, Robb *et al.*, 1972;  $15.9 \pm 2.0\%$  of empty BM, Westervelt *et al.*, 1976; <1 to >11% of Live BM, Webb and Weaver, 1979; 1-2% Live BM, Gunn, 1986; 10-24% of empty BM, Kane *et al.*, 1987; 2.6-14.7% of empty BM, Martin-Rosset *et al.*, 2008). The range of neutral body lipid contents recorded for these ponies, however, was within the wide range (1 to 60%) reported for various animal species (Siri, 1956; Lohman, 1971). In the present study, structural (polar) lipids comprised a constant <1% of recEBM, a value within the published range for man (Siri, 1956).

#### **4.6 Conclusion**

By using animals of common breed and sex, these data demonstrated an exponential relationship between linearly-ordinal BCS points and body fat content and suggested a loss of sensitivity of subjective BCS systems in overweight subjects. Objective measures of whole body adiposity, including measures of neck and body girths and ventral abdominal, retroperitoneal fat depth may provide useful supplements to BCS for monitoring live obese ponies. As for other species, further study on larger numbers of animals is warranted before data can be confidently extrapolated to animals of differing breeds, ages and sexes and to define 'healthy' ranges of body fat content (Russel *et al.*, 1969; Wright and Russel, 1984). The complex interactions between body fat, appetite and the gastro-intestinal tract are also of future interest.

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# **Supplementary Information 1**

## **SI 1.1 Details of cadaver dissection, sample preparation and additional results of analyses**

### ***Systematic cadaver dissection and sampling***

After exsanguination and collection of blood, removal of the head (atlanto-occipital joint dislocation), lower limbs (mid-carpal/tarsal dislocation) and tail (sacrococcygeal dislocation), the carcass hide (skin and pelage) was mechanically removed. Hide was also dissected from the head, lower limbs and tail. Muscle and fat remnants associated with the hide were separated and weighed along with the underlying site-specific soft tissue samples. Total hide mass (from carcass, head, lower limbs and tail, including pelage, mane and tail) was recorded ( $\pm 0.05\text{kg}$ , Salter mechanical bench scale model 250; Avery Weigh-Tronix, West Bromwich, West Midlands, UK) and twenty,  $2\text{cm}^2$ , shaven skin samples were collected from ten, anatomically pre-defined sites and stored ( $-20^\circ\text{C}$ ) in sealed plastic containers (to limit moisture loss) pending dicing and chemical analyses (Table 1).

The skinned carcass was eviscerated (thoracic and abdominal contents together) and the viscera (with their contents), including as much of the accompanying fluid (e.g. urine, peritoneal fluid) as possible were collected and weighed ( $\pm 0.5\text{kg}$ , Kern HTS 1.5T0.5 IP; Kern and Sohn GmbH, Balingen, Germany). Individual thoracic and abdominal viscera were separated and weighed. Total digesta mass was determined and the emptied gastrointestinal tract was rinsed with cold water and drained before being re-weighed. Organ associated adipose tissues were removed and weighed and the adipose-denuded weight of each organ was recorded ( $\pm 1\text{g}$  [Salter Electronic Kitchen Scale with timer and clock, Model 1014; Salter Housewares Ltd., Tonbridge UK], or  $\pm 10\text{g}$  [Weigh-Tronix, Capacity 25 x  $0.01\text{kg}$ ; Avery Weigh-Tronix Ltd., West Bromwich, West Midlands, UK] or  $\pm 0.05\text{kg}$  [Salter mechanical bench scale model 250; Avery Weigh-Tronix, West Bromwich, West Midlands, UK] or  $\pm 0.5\text{kg}$  [Kern HTS 1.5T0.5 IP; Kern and Sohn GmbH, Balingen, Germany] as appropriate). All thoracic and abdominal viscera along with their adipose tissues were then re-pooled, re-weighed and stored ( $-20^\circ\text{C}$ ) in sealed plastic bags pending mincing/homogenisation and chemical analyses. A sample ( $\sim 500\text{g}$ ) of thoroughly mixed digesta was also stored ( $-20^\circ\text{C}$ ) for later determination of dry matter content. The remaining empty carcass was divided sagittally into right and left halves, the spinal cord retrieved (to be stored at  $-20^\circ\text{C}$  with brain tissue) and each half carcass weighed ( $\pm 0.5\text{kg}$ ) before being divided into three sections to facilitate handling and storage ( $4^\circ\text{C}$ ) in sealed plastic bags prior to dissection. Right half carcass dissection was completed within 72hr of euthanasia whereas dissection and tissue category storage of: blood, viscera, head, lower limbs, dock, central nervous system (brain and spinal cord) and skin were completed within 12 hours of death.

The skinned head and lower limbs were weighed. The head was bisected sagittally (Butcher Boy band saw Model 1640; Butcher Boy UK Ltd., Ayrshire, Scotland) ensuring the tongue was equally divided. The whole brain was removed and weighed and stored ( $-20^\circ\text{C}$ ) with the spinal cord (CNS) as described previously. The right half-head was dissected to yield bone, muscle, adipose tissue (peri-orbital and 'other') and eyeball components which were weighed separately. The right lower forelimb and hindlimb were dissected into bone (including hoof capsules, from which

the digital cushions were not removed), muscle/tendon and adipose components, each of which was weighed separately. Soft tissues (muscle, tendon, adipose, eyeball) from the right half head and lower limbs were then pooled and frozen (-20°C) pending mincing/homogenisation and chemical analyses. Right half head and lower limb bones (including hoof capsules) were weighed, pooled together and frozen (-20°C) pending grinding and chemical analyses. The skinned left half head and lower limbs were weighed and were assumed to have the same bone and adipose tissue weights as the right halves, the difference representing non-bone/non-WAT soft tissues.

The soft tissues (subcutaneous adipose tissue and muscles and ligaments) of the dock were dissected from the bones. The right side muscles/ligaments and subcutaneous adipose tissues were weighed and later frozen with the remainder of the right side soft tissues (see below). Half the mass of coccygeal vertebrae was pooled with the remaining right side body bones (see below).

The three portions of the right side of the carcass (body wall, neck and upper limbs) were deboned by a professional butcher so as to remove as much of the attached soft tissues as possible. The bones (including costal, sternal, scapular and pedal bone lateral cartilages, but not joint menisci) including half the mass of the dock bones, were finally weighed and stored (-20°C) pending grinding and chemical analyses. (Menisci were removed with the peri-articular ligaments and stored with the other soft tissues). Muscles/tendons/ligaments and regional white adipose tissues (superficial/subcutaneous, inter-muscular & peri-articular and deep/intracavitary) of the right half carcass were dissected and weighed separately before being re-pooled, including the right side dock soft tissues, and frozen (-20°C) pending mincing and chemical analyses. (Intra-muscular white adipose tissue could not be separated.) Right and left carcass halves differed by only  $0.9 \pm 0.6\text{kg}$ , so the left half carcass was considered to have the same bone and adipose tissue masses as the right half, the remaining weight difference being attributed to non-bone/non-WAT soft tissues.

**Table SI 1.1:** Location of sites for collection of skin samples.

Site for skin sampling	No. samples taken
Centre of cheek, over masseter muscle	2
Lateral neck midway between crest and ventral midline and midway between head and shoulder	2
Lateral shoulder	2
Lateral thigh	2
Lateral proximal limbs: just proximal to carpus (forelimb) and hock (hindlimb)	2
Lateral distal limb, mid-cannon region (forelimb and hindlimb)	2
Lateral chest wall at mid-chest level	2
Dorsal midline at thoracolumbar region	2
Axillary region (glabrous skin)	2
Inguinal region (glabrous skin)	2

### ***Tissue homogenisation and proximate analyses***

#### ***i. Initial preparation of cadaver parts for chemical analyses***

Frozen bones were cut into pieces of roughly 2 square centimetres (Butcher Boy band saw Model 1640; Butcher Boy UK Ltd., Ayrshire, Scotland) to facilitate later grinding. Bone pieces and bone ‘shavings’ were re-frozen (-20°C) until grinding was possible.

Skeletally-associated (somatic) soft tissues from the right side of the carcass were par-thawed before being independently minced (Wolfking; Wolfking, Slagelse, Denmark): twice through a 13mm-hole plate and then twice through a 4mm-hole plate. Viscera and their associated WAT were similarly par-thawed and minced. Soft tissues from the right half head and right lower limbs were also par-thawed before being minced (Hobart; Hobart UK, Southgate, London): twice through a 9mm-hole plate and twice through a 5mm-hole plate. Minced tissues were thoroughly mixed between mincings and again after the final mincing, after which two representative samples (~500g) were collected and stored (-20°C) prior to final homogenisation and chemical analyses.

Bones from the right carcass and right half head plus right lower limbs were ground separately (Karl Schnell Mince Master; Karl Schnell Machine Works, Winterbach, Schorndorf, Germany): three times through a 12mm-hole plate. Ground bones were thoroughly mixed between grindings and again after the final grinding, after which two representative samples (~500g) were stored (-20°C) prior to final milling and chemical analyses.

## *ii. Final preparation of tissues and chemical analyses*

Frozen skin samples were diced (~1mm square). Blood, CNS and all minced soft tissue samples were homogenised separately for each pony (Moulinex Moulinette S; Group SEB, Ecully, France). Bone samples were ground to <1mm particles (Tecator grinding mill; Tecator AB, Perstorp, Sweden) and mixed thoroughly.

Standard proximate analytical techniques were then applied to triplicate (for moisture and gross energy determination) or duplicate (for lipid extraction, nitrogen analysis and ash determination) sub-samples of the homogenates (AOAC International, 2000). Samples (~10g) were freeze-dried (Edwards Modulyo and Speedivac Edwards High Vacuum pump; Edwards UK, West Sussex, UK) to enable calculation of sample dry matter. Dry samples (1 to 5g) were heated (at 550°C) to constant mass in a muffle furnace (Carbolite OAF 1; Carbolite Furnaces Ltd., Sheffield, UK), to determine ash content. Further dry samples (0.1 to 0.5g) were subjected to isothermal bomb calorimetry to determine the gross energy content (E2K Combustion Calorimeter; Digital Data Systems (Pty), Ltd., Northcliff, South Africa; Distributed by Sartec, Ltd., Tenterden, Kent, UK). Soxhlet petroleum ether extraction (Büchi extraction system B-811; Büchi Labortechnik AG, Postfach, Switzerland), of dry samples (1 to 2g) both before and after hydrochloric acid hydrolysis enabled quantification of non-polar/neutral lipid (mainly triglycerides) and total lipid (polar and non-polar lipids) respectively (AOAC International, 2000). Nitrogen content of fresh samples (~3g), later corrected to dry matter, was determined by the Kjeldahl titration technique (Büchi digestion unit K-435 and Büchi distillation unit B-324; Büchi Labortechnik AG, Postfach, Switzerland, and Titration manager TIM 845; Radiometer Analytical, Lyon, France), enabling quantification of sample crude protein ( $N \times 6.25 = \text{protein}$ ) (AOAC International, 2000).

**Table SI 1.2:** The mass (g) of each white adipose tissue (WAT) depot is presented, for seven mature pony mares of increasing body condition score (BCS system: 1 [very poor] to 9 [extremely fat]).

Pony BCS	1	2	3	4	5	6	7	mean	se
<b>a) Internal carcass (body wall-associated)</b>									
Intra-thoracic	80	326	362	576	814	960	1160	<b>611</b>	146
Intra-abdominal	0	506	5510	2430	7884	14632	9668	<b>5804</b>	2014
Intra-pelvic	66	206	156	3179	1578	464	1232	<b>983</b>	426
<b>Total (a)</b>	<b>146</b>	<b>1038</b>	<b>6028</b>	<b>6185</b>	<b>10276</b>	<b>16056</b>	<b>12060</b>	<b>7398</b>	2190
<b>b) Internal carcass (organ-associated)</b>									
Omental	70	91	505	105	2554	1468	885	<b>811</b>	350
Mesenteric	879	631	2585	1237	8150	8981	6574	<b>4148</b>	1373
Perirenal	42	105	295	351	471	430	425	<b>303</b>	63
Pericardial	109	155	153	287	298	107	563	<b>239</b>	62
Epicardial	52	95	85	257	165	116	301	<b>153</b>	35
Uterine broad lig	0	224	400	470	646	1080	600	<b>489</b>	129
<b>Total (b)</b>	<b>1152</b>	<b>1301</b>	<b>4023</b>	<b>2707</b>	<b>12284</b>	<b>12182</b>	<b>9348</b>	<b>6142</b>	1884
<b>Total Internal (a+b)</b>	<b>1298</b>	<b>2339</b>	<b>10051</b>	<b>8892</b>	<b>22560</b>	<b>28238</b>	<b>21408</b>	<b>13541</b>	3989
<b>c) External carcass (palpable)</b>									
Subcutaneous	45	1046	2346	1792	3980	11284	10762	<b>4465</b>	1754
Udder-associated	17	10	200	0	210	736	838	<b>287</b>	134
Nuchal	16	1144	718	673	620	3065	2794	<b>1290</b>	442
Inter-muscular	832	1504	2838	10280	15480	15620	18180	<b>9248</b>	2812
<b>Total External (c)</b>	<b>910</b>	<b>3704</b>	<b>6102</b>	<b>12745</b>	<b>20290</b>	<b>30705</b>	<b>32574</b>	<b>15290</b>	4863
<b>d) Head/lower limb</b>									
Peri-orbital	96	120	98	132	118	90	132	<b>112</b>	7
Other	26	232	150	308	180	234	352	<b>212</b>	41
<b>Total Extremity (d)</b>	<b>122</b>	<b>352</b>	<b>248</b>	<b>440</b>	<b>298</b>	<b>324</b>	<b>484</b>	<b>324</b>	46
<b>GRAND TOTAL (a+b+c+d)</b>	<b>2330</b>	<b>6395</b>	<b>16401</b>	<b>22077</b>	<b>43148</b>	<b>59267</b>	<b>54466</b>	<b>29155</b>	8720
Recovered Empty Body Mass	129738	141831	179490	212006	191481	198263	243212	<b>185146</b>	14867
<b>Lean Empty Body Mass</b> (includes intramuscular WAT)	<b>127408</b>	<b>135436</b>	<b>163089</b>	<b>189929</b>	<b>148333</b>	<b>138996</b>	<b>188746</b>	<b>155991</b>	9589

**Table SI 1.3:** Organ/tissue masses (g) from seven pony mares ranked according to increasing body condition score. Values presented in brackets below each mass represent that mass as a proportion of WAT-free recovered empty body mass.

<b>Pony</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>mean</b>	<b>se</b>
<b>Body Condition Score</b>	1.25	2.5	4.08	4.25	5.9	6.8	7		
<b>CNS</b>	620 (0.005)	627 (0.005)	680 (0.004)	646 (0.003)	651 (0.004)	709 (0.005)	652 (0.003)	<b>655</b> (0.004)	12 (0.000)
<b>Respiratory tract</b>	1898 (0.015)	1793 (0.013)	2462 (0.015)	2536 (0.013)	1798 (0.012)	1634 (0.012)	2193 (0.012)	<b>2045</b> (0.013)	134 (0.001)
<b>Heart</b>	1142 (0.009)	1015 (0.007)	1395 (0.009)	1595 (0.008)	1205 (0.008)	1225 (0.009)	1535 (0.008)	<b>1302</b> (0.008)	80 (0.000)
<b>GI tract</b>	11516 (0.090)	11050 (0.082)	11150 (0.068)	14712 (0.077)	9160 (0.062)	9000 (0.065)	10500 (0.056)	<b>11013</b> (0.072)	719 (0.005)
<b>Pancreas</b>	170 (0.001)	not avail	not avail	not avail	224 (0.002)	274 (0.002)	251 (0.001)	<b>230</b> (0.001)	17 (0.000)
<b>Liver</b>	2330 (0.018)	1785 (0.013)	2645 (0.016)	3785 (0.020)	2505 (0.017)	1365 (0.010)	3495 (0.019)	<b>2559</b> (0.016)	326 (0.001)
<b>Spleen</b>	271 (0.002)	445 (0.003)	545 (0.003)	765 (0.004)	535 (0.004)	421 (0.003)	805 (0.004)	<b>541</b> (0.003)	72 (0.000)
<b>Kidneys</b>	649 (0.005)	755 (0.006)	665 (0.004)	913 (0.005)	694 (0.005)	677 (0.005)	564 (0.003)	<b>702</b> (0.005)	41 (0.000)
<b>Uterus/bladder</b>	789 (0.006)	310 (0.002)	825 (0.005)	547 (0.003)	429 (0.003)	1410 (0.010)	1295 (0.007)	<b>801</b> (0.005)	159 (0.001)

**Table SI 1.4:** Results of proximate analysis for the eight tissue categories from each of seven mature pony mares, ranked by increasing body condition score.

BODY SOFT TISSUES							
pony	% H <sub>2</sub> O	On dry matter basis					GE (MJ/kg)
		% NL	% TL	% N	% CP	% Ash	
1	78.05	0.76	3.12	14.49	90.56	3.60	25.169
2	71.96	18.28	20.03	11.55	72.19	2.21	29.645
3	66.92	35.14	37.23	9.07	56.69	2.64	30.769
4	67.67	32.51	34.62	9.57	59.81	1.60	28.907
5	55.5	60.64	61.42	5.49	34.31	0.66	30.758
6	45.3	68.94	71.12	3.92	24.50	0.55	33.621
7	55.51	61.01	61.85	5.56	34.75	1.01	31.535
BODY BONES							
pony	% H <sub>2</sub> O	On dry matter basis					GE (MJ/kg)
		% NL	% TL	% N	% CP	% Ash	
1	50.96	6.22	7.48	6.40	40.00	48.49	10.968
2	35.56	23.05	24.30	5.21	32.56	40.97	15.508
3	29.58	22.33	23.80	4.91	30.69	39.37	15.293
4	20.43	26.05	26.72	3.80	23.75	43.80	15.097
5	27.13	31.57	32.50	4.43	27.69	34.96	17.972
6	25.03	25.34	26.00	4.58	28.63	41.30	15.735
7	25.65	27.41	28.39	4.56	28.50	38.99	16.607
VISCERA							
pony	% H <sub>2</sub> O	On dry matter basis					GE (MJ/kg)
		% NL	% TL	% N	% CP	% Ash	
1	82.05	3.07	5.91	12.42	77.63	4.93	20.328
2	80.97	25.18	26.18	9.39	58.69	3.38	23.685
3	68.75	44.39	45.9	6.76	42.25	1.86	25.942
4	72.34	31.79	33.49	8.35	52.19	3.66	24.694
5	53.42	69.85	70.79	3.34	20.88	0.94	32.257
6	49.46	71.99	72.75	3.13	19.56	1.05	32.595
7	59.62	60.06	60.82	4.61	28.81	0.60	29.977
HIDE							
pony	% H <sub>2</sub> O	On dry matter basis					GE (MJ/kg)
		% NL	% TL	% N	% CP	% Ash	
1	67.47	1.98	2.86	16.02	100.13	2.73	21.625
2	56.53	2.03	2.83	17.43	108.94	2.04	22.313
3	57.09	2.71	3.56	15.90	99.38	2.51	21.710
4	60.92	2.48	3.56	17.52	109.50	2.54	21.904
5	67.94	4.38	4.60	15.48	96.75	3.03	21.729
6	61.40	6.30	7.05	14.03	87.69	2.75	22.500
7	64.48	3.48	4.25	15.98	99.88	2.41	22.220

HEAD / LOWER LIMB SOFT TISSUES							
pony	% H <sub>2</sub> O	On dry matter basis					GE (MJ/kg)
		% NL	% TL	% N	% CP	% Ash	
1	76.87	7.00	9.64	14.17	88.56	3.09	24.829
2	71.72	28.18	30.35	11.06	69.13	1.77	27.886
3	70.94	25.84	28.44	11.26	70.38	1.89	28.681
4	68.70	32.33	34.91	10.15	63.44	1.35	28.870
5	69.51	32.94	34.71	10.01	62.56	1.82	29.470
6	67.41	34.86	37.82	9.37	58.56	1.29	27.043
7	68.78	34.21	36.03	9.84	61.50	1.51	26.129
HEAD / LOWER LIMB BONES							
pony	% H <sub>2</sub> O	On dry matter basis					GE (MJ/kg)
		% NL	% TL	% N	% CP	% Ash	
1	39.16	1.76	3.51	6.74	42.13	49.50	12.460
2	25.60	12.73	13.46	5.38	33.63	49.27	12.859
3	25.61	10.94	11.79	5.54	34.63	46.40	12.480
4	24.54	16.10	16.90	5.74	35.88	44.23	12.875
5	23.55	19.32	19.92	5.81	36.31	40.58	14.000
6	23.19	12.19	12.85	5.84	36.50	47.28	12.495
7	24.40	11.31	12.10	6.66	41.63	42.72	14.014
CNS							
pony	% H <sub>2</sub> O	On dry matter basis					GE (MJ/kg)
		% NL	% TL	% N	% CP	% Ash	
1	73.73	47.23	54.39	6.54	40.88	5.41	29.273
2	75.18	49.93	55.38	6.53	40.81	5.82	28.500
3	71.17	51.78	52.98	5.69	35.56	5.43	29.306
4	72.59	47.12	58.01	6.27	39.19	5.93	29.773
5	72.57	49.97	55.49	6.22	38.88	5.59	29.608
6	72.34	50.30	56.94	6.78	42.38	5.86	29.301
7	71.14	47.34	57.02	6.01	37.56	5.82	30.143
BLOOD							
pony	% H <sub>2</sub> O	On dry matter basis					GE (MJ/kg)
		% NL	% TL	% N	% CP	% Ash	
1	80.81	0.39	0.72	15.28	95.50	4.12	23.473
2	82.78	0.45	1.15	14.75	92.19	4.40	23.093
3	78.02	0.28	0.61	15.41	96.31	3.58	23.407
4	78.72	0.33	0.46	15.38	96.13	3.69	22.906
5	77.15	0.31	0.37	15.29	95.56	3.47	23.770
6	75.77	0.39	0.50	15.28	95.50	3.81	23.577
7	78.54	0.38	0.67	15.22	95.13	3.75	23.401



**Table SI 1.5:** Individual muscle, visceral and white adipose tissue depot water contents, presented as percentages for 6 of 7 mature pony mares of differing body condition scores.

Pony	BCS	<i>Muscles</i>				<i>Viscera</i>	
		Middle Gluteal	Semimembranosus	Diaphragm	Cardiac	Liver	Kidney
1	1.25	77.66	77.08	79.05	80.91	68.11	80.42
2	2.5						
3	4.08	73.98	75.00	77.09	79.08	69.93	80.39
4	4.25	73.95	75.89	75.55	78.03	67.58	81.59
5	5.9	71.61	75.15	70.81	78.75	67.17	80.09
6	6.8	68.11	71.45	64.81	78.15	65.86	79.62
7	7	72.85	72.88	72.11	77.80	69.56	79.05

Pony	BCS	<i>White adipose depots</i>									
		Tailhead	Lateral withers	Nuchal crest	Ventral abdominal retroperitoneal	Intrapelvic	Omental	Mesenteric	Perirenal	Epicardial	Pericardial
1	1.25	72.58		76.27		87.7	87.21	86.99	90.11	89.01	
2	2.5										
3	4.08	24.62	34.72	22.56	11.16	8.55	16.25	22.92	13.26	16.04	9.64
4	4.25	30.21	24.75	47.92	9.14	5.15	20.37	16.39	7.35	7.33	8.23
5	5.9	12.41	10.93	9.96	5.61	4.42	18.67	4.18	6.12	7.60	7.51
6	6.8	13.56	6.92	10.93	4.49	6.94	11.13	7.52	5.80	9.99	6.35
7	7	16.75	15.85	10.84	4.83	4.39	7.06	6.11	10.03	6.29	9.87

**Table SI 1.6:** Gross energy contents (MJ/kgDM) of muscle, visceral and white adipose tissues from 6 of 7 mature pony mares of differing body condition scores. \*Only 1 sample tested; the result was excluded from calculation of the mean value.

		<i>Muscles</i>				<i>Viscera</i>					
Pony	BCS	Middle Gluteal	Semimembranosus	Diaphragm	Heart	Liver	Kidney				
1	1.25	23.06	25.46	26.95	25.82	20.14	22.33				
2	2.5										
3	4.08	25.33	25.54	25.42	24.23	22.18	22.40				
4	4.25	28.45	20.23		26.17	21.98	20.40				
5	5.9	29.27	25.30	30.08	27.57	25.81	28.36				
6	6.8	26.78	26.29	32.11	28.26	22.05	20.53				
7	7	25.51	24.87	27.58	24.20	25.81	21.51				
mean		26.40	24.61	28.43	26.04	23.00	22.59				
se		0.93	0.90	1.19	0.68	0.94	1.21				
<i>White adipose depots</i>											
		Ventral									
		abdominal									
		retroperitoneal									
Pony	BCS	Tailhead	Withers	Nuchal	Intra-pelvic	Omental	Mesenteric	Peri-renal	Epi-cardial	Peri-cardial	
1	1.25	*27.639									
2	2.5										
3	4.08	41.120	40.304	43.231	41.266	41.182	39.521	40.806	40.798	41.391	41.059
4	4.25	41.795	43.519	38.223	42.547	44.738	46.595	42.078	43.794	42.506	41.471
5	5.9	44.694	43.103	42.173	45.762	43.198	40.317	43.339	41.818	43.359	40.613
6	6.8	41.697	42.732	39.321	41.393	40.918	41.151	41.759	43.444	42.925	42.262
7	7	41.513	41.290	41.335	42.263	41.756	40.874	40.815	41.008	41.381	44.094
mean		42.16	42.19	40.86	42.65	42.36	41.69	41.76	42.17	42.31	41.90
se		0.64	0.60	0.92	0.82	0.71	1.26	0.47	0.62	0.40	0.61

# Chapter 5

## **Assessment of body fat in the pony: II. Validation of the deuterium oxide dilution technique for the measurement of body fat**

During the writing of this thesis, this chapter has been accepted for publication:

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Preliminary data from this chapter were also presented as an abstract and a poster:

Dugdale, A., Curtis, G., Harris, P.A., Milne, E., Argo, C.McG. (2010) Validation of the D<sub>2</sub>O dilution method for measurement of body fat content in living ponies.

- a) In: The impact of nutrition on the health and welfare of horses. Proceedings of the 5<sup>th</sup> European Workshop on Equine Nutrition, Cirencester, UK, September 2010. The European Association for Animal Production publication No. 128. Eds: Ellis AD, Longland AC, Coenen M, Miraglia N. P 235-6.
- b) Proceedings of the 49<sup>th</sup> British Equine Veterinary Association Congress, Birmingham, UK, September 2010. P 234.

## 5.1 Summary

Excessive accumulations or depletions of body fat have been associated with increased morbidity and mortality in horses and ponies. An objective, minimally-invasive method to accurately quantify body fat in living animals is required to aid nutritional management and define welfare/performance limits. We aimed to compare deuterium oxide (D<sub>2</sub>O) dilution-derived estimates of total body water (TBW) and body fat with values obtained by ‘gold standard’ proximate analysis and cadaver dissection. Our hypothesis was that D<sub>2</sub>O dilution offers a valid method for the determination of TBW and body fat in *Equidae*.

Seven mature (13 ±3yr, 212 ±14kg, body condition scores 1.25 to 7/9), healthy, Welsh Mountain pony mares, destined for euthanasia (for non-research purposes) were used. (These were the same animals as those reported in Chapter 4.) Blood samples were collected before and 4h after D<sub>2</sub>O (0.11-0.13g/kg, 99.8 atom percent excess) administration. Filtered plasma was analysed by gas isotope ratio mass spectrometry following zinc reduction of hydrogen isotopes. After euthanasia, white adipose tissue (WAT) mass was recorded before all body tissues were analysed by proximate chemical analyses.

D<sub>2</sub>O dilution-derived estimates of TBW and body fat were strongly associated with proximate analysis- and dissection-derived values (all  $R^2 > 0.97$ ,  $P \leq 0.0001$ ). Bland-Altman analyses demonstrated good agreements between methods. D<sub>2</sub>O dilution slightly over-estimated TBW (0.79%, Limits of Agreement (LoA) -3.75 to 2.17%) and under-estimated total body lipid (1.78%, LoA -0.59 to 4.15%) and dissected WAT (0.72%, LoA -2.77 to 4.21%).

This study provides the first validation of the D<sub>2</sub>O dilution method for the minimally-invasive, accurate, repeatable and objective measurement of body water and fat in living *Equidae*.

## 5.2 Introduction

For man and other domestic species, total body fat mass (TBFM) has been associated with general and reproductive health, welfare and athletic performance (Morrow, 1976; Barr *et al.*, 1994; Henneke *et al.*, 1983; Sillence *et al.*, 2006). For living horses and ponies, for which obesity and emaciation are common causes of morbidity and mortality (Hintz, 1999; Sillence *et al.*, 2006), valid methods for the accurate quantification of TBFM have yet to be identified. The sheer size of these animals precludes the use of methods such as body density measurement and dual energy X-ray absorptiometry analysis, which have been invaluable for smaller species including man (Brodie *et al.*, 1998; Speakman *et al.*, 2001a).

Traditionally, accurate and precise measurements of TBFM have been based on invasive, destructive and often expensive techniques which are inappropriate for practical use in living animals when repeated measures are often required to monitor changes in body composition (Johnson and Nagy, 2005; Wells and Fewtrell, 2006). However, when published data describing the ‘fat’ contents of domestic animals are reviewed, complications are encountered on several fronts. Total body ‘fat’ has variously been defined as dissected white adipose tissue (WAT, which excludes intramuscular fat deposits), as chemically extracted lipid (which, dependent on analytical methodology, may comprise total lipid or only neutral lipid) or as TBFM, retrospectively calculated after determination of the body water pool size using indicator dilution methods (Wang *et al.*, 1992). Whereas proximate analysis of body lipids generally describes the neutral lipid content, mostly storage triglycerides associated with adipocytes, following petroleum ether extraction, the use of different solvents and extraction techniques enables determination of total lipid content, which

includes not only the neutral storage lipids but also the compound structural lipids that are essential for normal body function and include sphingolipids and phospholipids (Wang *et al.*, 1992). Although the direct evaluation of body composition should offer 'gold standard' measurements of body 'fat', comparison of values derived from different studies is further confounded by the extent of the body investigated; whole body, exsanguinated body, eviscerated body with or without the head, hide or distal limbs, half carcass or 12<sup>th</sup> rib section (Robb *et al.*, 1972; Webb and Weaver, 1979; Lunt *et al.*, 1985; Andrew *et al.*, 1994).

Tracer dilution methodologies for the estimation of TBFM have been validated against post mortem-derived measures in other species and could usefully be applied to living horses and ponies (Houseman *et al.*, 1973; Odwongo *et al.*, 1984; Burkholder and Thatcher, 1998). These methods capitalize on the inverse relationship between body contents of water and fat (Siri, 1956). Total body water (TBW) pool size must first be quantified by administration of a known dose of tracer into one of the body fluid compartments and the determination of the extent of its dilution after equilibration throughout the TBW pool (Speakman *et al.*, 2001b). TBW measurement (hydrometry) accuracy is optimised if the tracer can integrate with normal body water such that isotopes of hydrogen and oxygen are preferred. Practical and economic constraints have made deuterium ( $D_2$ ), a stable isotope of hydrogen, the most commonly used substance for this purpose when administered as deuterium oxide ( $D_2O$ ) (Speakman *et al.*, 2001b; Wells and Fewtrell, 2006). Deuterium dilution techniques have been extensively used for the estimation of TBW and TBFM in various mammalian species, however, its application for the estimation of either of

these variables in horses has yet to be validated (Schoeller *et al.*, 1980; Andrews *et al.*, 1997; Burkholder and Thatcher, 1998; Forro *et al.*, 2000; Speakman *et al.*, 2001b).

An ability to quantify the TBFM of living horses would empower clinical and epidemiological investigation of the associations between body fat, health and performance. This study tested the agreement between measures of TBW and TBFM generated in living animals by the minimally invasive, deuterium oxide dilution method with ‘gold standard’, post mortem measurements of i) body lipid determined by chemical (proximate) composition analysis and ii) WAT determined by dissection.



### 5.3 Materials and Methods

#### *Animals*

Seven mature ( $13 \pm 3$ yr [range 6 to 20yr],  $212 \pm 14$ kg), Welsh Mountain pony mares, ranging between body condition scores (BCS) 1.25/9 ('very poor') and 7/9 ('fleshy'), which had been designated for euthanasia (for non-research reasons) were donated by their owners for inclusion in the study (Table 1). Owner election for euthanasia was prompted by various chronic conditions in the animals which had proved refractory to treatment (dental disease [P1 & P2], sinusitis [P3], bilateral blindness secondary to uveitis [P4], non-healing facial fistula following dental extraction [P5], infertility [P6], epilepsy [P7]). Five of the seven ponies had been fed an identical forage-based diet for 10 days prior to euthanasia. These ponies were the same as those reported in Chapter 4.

#### *Deuterium oxide dilution*

On the day prior to euthanasia (09:00h to 10:00h), each pony was weighed ( $\pm 1$ kg, Lightweight Intermediate weigh scales; HorseWeigh, Llandrindod Wells UK; calibration checked regularly) and body condition scored (BCS) in accordance with criteria described by Kohnke (1992, a modification of Henneke *et al.*, 1983). A jugular intravenous catheter (12g, 80mm PTFE catheter, Intraflon 2; Vygon, Ecouen, France) was then placed percutaneously under aseptic conditions and local anaesthesia to facilitate deuterium oxide (D<sub>2</sub>O) administration.

Doses of D<sub>2</sub>O (99.8 atom percent excess (APE); supplied by CK Gas Products, Hook, Hampshire, UK; manufactured by Cambridge Isotope Laboratories Inc., Andover, MA, USA) were individually calculated in accordance with each animal's

BM and BCS to partially compensate for gross differences in the overall hydration of body tissues in order to promote equality of deuterium enrichments in body water between animals of different gross BCS classifications (obese, moderate, thin), to optimise subsequent deuterium measurement accuracy (Lobley G., Personal Communication). Doses were 0.11g/kg (obese, BCS 7 to 9/9, n = 2), 0.12g/kg (moderate BCS 4 to 6/9, n = 3) and 0.13g/kg (thin, BCS 1 to 3/9, n = 2).

Blood samples (20ml) were collected by venepuncture (Lithium heparin BD Vacutainer; Becton, Dickinson and Co., New Jersey, USA) from the contralateral jugular vein immediately before and 4h after D<sub>2</sub>O administration (deuterium equilibration time determined by Fuller *et al.*, 2004). Each dose of D<sub>2</sub>O was administered via the jugular venous catheter over 15-60 seconds and immediately followed by 100ml infusion of sterile saline to maximise delivery of the deuterium dose. Syringes used to deliver the D<sub>2</sub>O were re-weighed immediately post-infusion and the actual mass of infused D<sub>2</sub>O ( $\pm 0.1$ mg, Ohaus GT480; Ohaus Corporation, Pine Brook, New Jersey, USA) was determined by difference. Food and water were withheld from the animals during the 4h D<sub>2</sub>O equilibration period.

Plasma was immediately harvested from each blood sample (10min, 2000g at 4°C, Hermle Z 300 K; Hermle Labortechnik GmbH, Wehingen, Germany) and stored in 1-2ml aliquots in air-tight tubes (Safe-T-Seal tubes and Screw Cap with Gasket; Fisherbrand, Thermo Fisher Scientific Oy, Vantaa, Finland) at -80°C pending analysis. Thawed plasma samples were filtered (Costar Spin-X centrifuge tube with 0.22µm nylon filter, Corning Inc, New York, USA) and analysed in triplicate by gas isotope ratio mass spectrometry (Sira 10, VG Isotech, Cheshire, UK) following zinc

reduction of the hydrogen isotopes present in the plasma water (Fuller *et al.*'s (2004) and Midwood's (1990) modifications of the methods described by Wong *et al.*, 1987). (Further details are presented in the supplementary file (SI 2) at the end of this chapter.) Deuterium abundances (expressed as parts per thousand different from reference,  $\delta$  ‰) in plasma samples were determined relative to a reference water (Aberdeen tap water) which had previously been normalised against the international standards V-SMOW and SLAP according to Gonfiantini (1978). Deuterium abundances ( $\delta$  ‰) were converted to parts per million:-

$$D_2 \text{ ppm} = (0.15579 \times \text{normalised } \delta \text{ (‰)}) + 155.79 \quad (1)$$

where, for example, 155.79 is the  $D_2$  abundance of V-SMOW. Measurement CVs were <0.5% for the mean parts per million values in plasma. Calculation of the dilution space available to deuterium oxide ( $D_2O$  space) was as follows:-

$$D_2O \text{ space (g)} = \frac{(\text{Dose (g)} \times P_b \text{ amu})}{\text{Dose amu}} \times \frac{(\text{Dose ppm} - P_b \text{ ppm})}{(P_e \text{ ppm} - P_b \text{ ppm})} \quad (2)$$

where,  $P_b$  is baseline plasma;  $P_e$  is equilibrium plasma; Dose ppm is 998,000 (99.8 APE, confirmed by analysis of several dilutions); Dose amu (atomic mass units) is the molar mass of the stock solution of 99.8 APE deuterium oxide (= 20.02g/mole);  $P_b$  amu was calculated for each pony according to its measured deuterium abundance and an assumed constant  $^{18}O$  abundance of 0.2% (the value was approx. 18.0148g/mole for all ponies). A 4% correction factor was applied to the calculated  $D_2O$  space to account for isotopic exchange of deuterium with non-water hydrogen present in proteins, carbohydrates and possibly fats (Racette *et al.*, 1994), such that:-

$$\text{Corrected D}_2\text{O space (TBW}_D) = \text{D}_2\text{O space}/1.04 \quad (3)$$

where, TBW<sub>D</sub> is the finally determined total body water mass (kg) according to deuterium oxide dilution. The actual BM recorded on the day of the D<sub>2</sub>O dilution study (BM<sub>D</sub>) was used to determine the percentage of total body tissues which was comprised of water. Lastly, the percentage of total body fat could be calculated. This final adjustment is dependent on the fact that stored triglycerides are anhydrous and application of the ‘universal’ hydration factor for lean tissues (0.732) suggested by Pace and Rathbun (1945):-

$$\text{Percentage Body Fat} = 100 - (\% \text{TBW}_D/0.732) \quad (4)$$

Total body fat mass (TBFM) was determined from the recorded BM on the day of D<sub>2</sub>O dilution (BM<sub>D</sub>). Alternatively, fat-free body mass (FFBM) is calculated as follows:-

$$\text{Fat-free body mass (FFBM)} = \frac{\text{TBW}_D(\text{kg})}{0.732} \quad (5)$$

and TBFM is then determined as the difference between BM<sub>D</sub> and FFBM.

Verification of isotope enrichment of the deuterium oxide used was performed during triplicate analyses of five dilutions of the stock solution (99.8 APE) with Aberdeen tap water, to result in final enrichments of the order of 300ppm, similar to those measured in the plasma samples.

### ***Cadaver dissection and proximate analysis***

Body mass was again recorded prior to euthanasia ( $BM_{PA}$ ) by intra-cranial free-bullet ( $n = 6$ ) or intravenous barbiturate overdose ( $n = 1$ ). Exsanguination was conducted immediately post mortem and blood was collected, weighed ( $\pm 10g$ ; Weigh-Tronix, Capacity 25 x 0.01kg; Avery Weigh-Tronix Ltd., West Bromwich, West Midlands, UK) and duplicate samples ( $\sim 500g$ ) stored ( $-20^{\circ}C$ ) in sealed plastic containers (to minimise evaporative losses or condensation gains) pending chemical analyses.

All cadavers were systematically dissected (as described in the supplementary files to Part I, Dugdale *et al.*, In Press) to yield eight discrete tissue categories: hide (including pelage); central nervous system (CNS: brain and spinal cord); collected blood; right side carcass (neck, body wall and upper limbs) bones; right half head and lower limb bones (including hoof capsules); right side carcass soft tissues (white adipose tissue [WAT], skeletal muscle and other); right half head and lower limb soft tissues (WAT and other); viscera (empty) with their associated WAT. White adipose tissue weights from the three soft tissue categories were recorded separately from the remaining tissues in those categories. Intramuscular white adipose tissue could not be separated. Peritoneal fluid and urine were collected and weighed. Total digesta mass was determined and the emptied gastrointestinal tract was washed with cold water and drained before being re-weighed with the remainder of the viscera. A sample ( $\sim 500g$ ) of thoroughly mixed gut contents (digesta) was stored ( $-20^{\circ}C$ ) for later determination of dry matter.

The left half body (left carcass, half head and lower fore- and hind-limbs), for which overall weights were recorded (pre-dissection left and right half-body masses differed by only  $0.9 \pm 0.6\text{kg}$ ), was not dissected but assumed to contain the same bone and adipose tissue masses as the dissected right half. The remaining mass was attributed to non-bone/non-WAT soft tissues. Differences between pre-euthanasia live weight and recovered body weight (sum of the eight tissue categories plus: gastrointestinal contents, urine, peritoneal fluid, skinned left body [left carcass, head and lower fore- and hind-limbs] bones and soft tissues) were assumed to be water, lost and/or gained by evaporation and/or condensation during dissection.

Pending processing and analysis, each tissue category was stored ( $-20^{\circ}\text{C}$ ) in sealed, tough polythene bags or containers to minimise evaporative losses or condensation gains. Each tissue category was par-thawed, variously minced or ground and thoroughly mixed before samples ( $\sim 500\text{g}$ ) were collected and homogenised for proximate analysis.

Standard proximate analytical techniques were conducted in triplicate (for moisture determination) or duplicate (for lipid extraction) for each homogenised tissue sample (AOAC International, 2000). Samples were freeze-dried (Edwards Modulyo and Speedivac Edwards High Vacuum pump; Edwards UK, West Sussex, UK) to enable calculation of sample dry matter rather than oven-dried in order to avoid sample combustion or the loss of volatile lipids which are present in high concentrations in horse adipose tissues (Robb *et al.*, 1972; Johnson and Nagy, 2005). Soxhlet petroleum ether extraction (Büchi extraction system B-811; Büchi Labortechnik AG, Postfach, Switzerland) of dry samples both before and after acid

hydrolysis, enabled quantification of neutral lipid (mainly triglycerides) and total lipid (neutral and polar lipids) respectively (AOAC International, 2000). Duplicate samples of gastrointestinal contents (~20g) were oven-dried (75°C) to constant mass to determine digesta hydration.

### ***Determination of body composition***

The chemical composition and total mass of each of the eight tissue categories enabled each individual pony to be ‘reconstructed’ in terms of its overall water, neutral lipid and total lipid content. For comparison with D<sub>2</sub>O dilution-derived estimates of *in vivo* total body water, proximate analysis-derived values for body water required that water contained in the gastro-intestinal and urogenital tracts and water lost or gained by evaporation and condensation during dissection were included in the latter measure. Peritoneal fluid proved impossible to quantify and no corrections for it were made.

The TBW mass was therefore determined independently for each animal by both proximate analysis (TBW<sub>PA</sub>) and following D<sub>2</sub>O dilution (TBW<sub>D</sub>). Proximate analyses enabled quantification of both neutral body lipid (NBL) and total body lipid (TBL), whereas gross white adipose tissue (WAT) was quantified by dissection. The D<sub>2</sub>O dilution technique allowed the derivation of total body fat mass (TBFM) (Equation 5). Unavoidably, dissected WAT would have contained some connective, neurovascular and lymphoid tissues although intramuscular WAT, even when visible as marbling within muscle bellies, was not separable. Structural/essential lipid was calculated by difference (TBL minus NBL).

### ***Data analysis***

All data were initially entered into Excel spreadsheets (Microsoft Office Professional Edition 2003; Microsoft Corp., Washington, U.S.A.) and statistical analyses were performed using Excel, Minitab version 15.1.0 (Minitab Inc., Pennsylvania, U.S.A.) and STATA 10 (Stata/IC 10.1; Stata Corp., Texas, U.S.A.). Normality of all data sets for body mass ( $BM_{PA}$  and  $BM_D$ ), body water ( $TBW_{PA}$  and  $TBW_D$ ) and body lipid (total (TBL) or neutral (NBL), as determined by proximate analysis), body fat (TBFM, as determined by deuterium oxide dilution), and body WAT (as determined by dissection), whether expressed as actual weights or percentages of Live BM, was confirmed by both visual assessment of their frequency distributions and by Anderson-Darling normality tests. F tests were used to check for equal variance of data prior to least squares linear regression analyses. In addition, scatterplots of the data were performed to ensure that data distributions were suitable for the application of linear regression. The outcome and predictor variables tested were: proximate analysis results for body water or body lipid and  $D_2O$  dilution-derived measures of body water or body fat respectively. Dissection-derived measures of body WAT were alternative outcome variables for regression on  $D_2O$ -derived predictor variables. In addition, dissection-derived body WAT was regressed on proximate analysis results for body lipid to investigate the relationship between these two 'standard' techniques. Following linear regression, normal distribution of the residuals was confirmed using the Anderson-Darling test. Coefficients of determination ( $R^2$ ) and 95% confidence intervals are reported for the results of linear regression analyses. Summary statistics, unless otherwise stated, are reported as mean  $\pm$  standard error. Statistical significance was assumed if  $P < 0.05$ .



Bland Altman plots (Bland and Altman, 1986) were used to assess agreement between the different measurement techniques. However with only 7 ponies in total, the assessment of variability in the differences was limited such that they were assumed to be normally distributed for calculation of the bias (the mean difference between techniques) and the limits of agreement ( $\text{LoA} = \text{bias} \pm 1.96 \times \text{Standard Deviation of the differences}$ ). *A priori* limits of agreement were set at  $\pm 5\%$ .

## 5.4 Results

All data were secured for all animals in each body composition category evaluated (proximate analysis, deuterium oxide dilution and dissection, Table 5.1). Following D<sub>2</sub>O administration, plasma samples were collected to determine equilibrated isotope enrichments after  $246 \pm 3$  min. Only one animal (P7) differed markedly in BM (>3kg) between the time of the deuterium oxide study (BM<sub>D</sub>) and pre-euthanasia (BM<sub>PA</sub>) on the following day (Table 5.1).

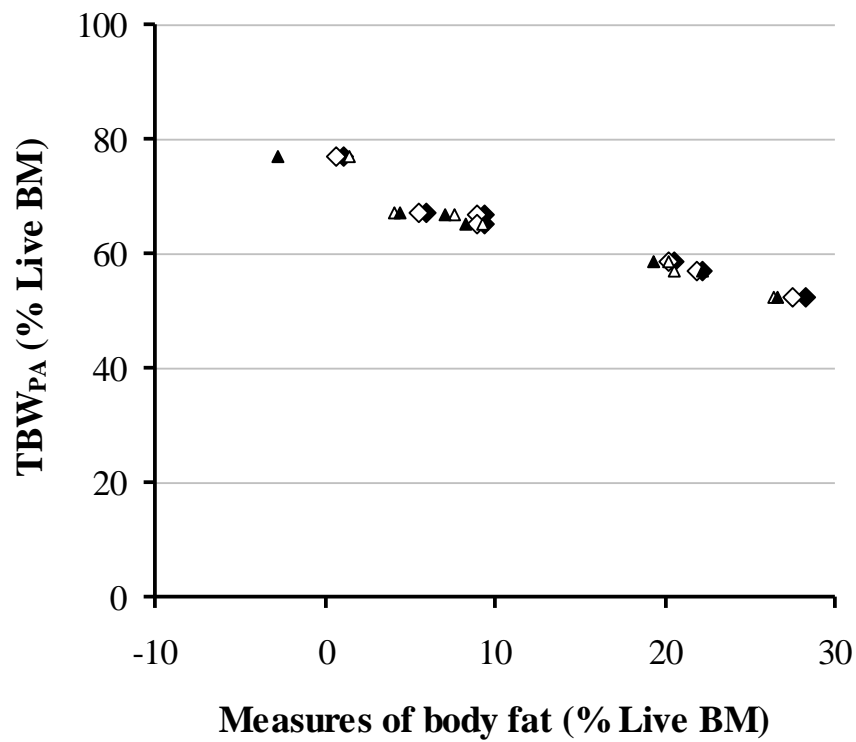
Proximate analysis-derived total body water (TBW<sub>PA</sub>) comprised between 52% BM<sub>PA</sub> (P6) and 77% BM<sub>PA</sub> (P1) and values varied inversely with all measures of body fat (Figure 5.1) and decreased linearly ( $R^2 = 0.88$ ) as BCS increased (Table 5.1). Following the recovery of digesta post mortem, it was determined that total digesta accounted for  $11.1 \pm 1.6\%$  (range 7.3 to 19.7%) of BM<sub>PA</sub> and digesta water comprised  $9.9 \pm 1.4\%$  (range 6.7 to 17.8%) of BM<sub>PA</sub> and  $15.2 \pm 1.4\%$  (range 12.2 to 23.1%) of TBW<sub>PA</sub>. Although the proportion of water contained in digesta was similar between animals ( $89.5 \pm 0.9\%$ ) regardless of BCS or BM<sub>PA</sub>, gut fill, measured as the relative proportions of BM<sub>PA</sub> comprised of digesta ( $R^2 = 0.91$ ) and of the TBW<sub>PA</sub> comprised of digesta water ( $R^2 = 0.81$ ; equivalent to a total of  $20.2 \pm 2.0$  kg gut water), decreased logarithmically as BCS increased. Water associated with urogenital tract contents ranged between 0 and 5 kg (0 - 2% of BM<sub>PA</sub>) and was independent of differences in BM or BCS.

**Table 5.1:** Summary data for the seven mature pony mares are presented in order of increasing body condition score (BCS, on a scale from 1 [very thin] to 9 [extremely fat]). Deuterium oxide dilution was performed on the day prior to euthanasia and subsequent dissection and proximate analysis. The body mass recorded prior to each procedure is provided ( $BM_D$  and  $BM_{PA}$ , respectively). Deuterium oxide dilution-derived values comprised total body water pool size ( $TBW_D$ ) and total body fat mass (TBFM). Proximate analysis-derived measures of total body water ( $TBW_{PA}$ ), total body lipid (TBL) and neutral body lipid (NBL) are also presented. Dissection enabled determination of white adipose tissue (WAT) mass and the water content of total digesta from each pony are presented.

Age (yr)	BCS (1-9)	D <sub>2</sub> O-dilution data			Proximate analysis data				Dissection data	
		$BM_D$ (kg)	$TBW_D$ (kg)	TBFM (kg)	$BM_{PA}$ (kg)	$TBW_{PA}$ (kg)	TBL (kg)	NBL (kg)	WAT (kg)	Digesta water (kg)
12	1.25	176	132.40	-4.88	173	133.44	1.75	1.07	2.33	30.76
9	2.5	160	111.64	6.98	159	106.84	9.39	8.72	6.40	17.17
11	4.08	215	146.38	15.03	216	144.45	20.26	19.13	16.40	21.98
17	4.25	241	161.83	19.92	238	155.45	22.35	21.10	22.08	19.00
20	5.9	211	120.20	46.79	211	120.11	46.69	45.98	43.15	18.17
6	6.8	226	121.50	60.02	225	117.95	63.47	61.68	59.27	14.66
16	7	279	164.95	53.65	270	158.84	55.44	54.50	54.47	19.76

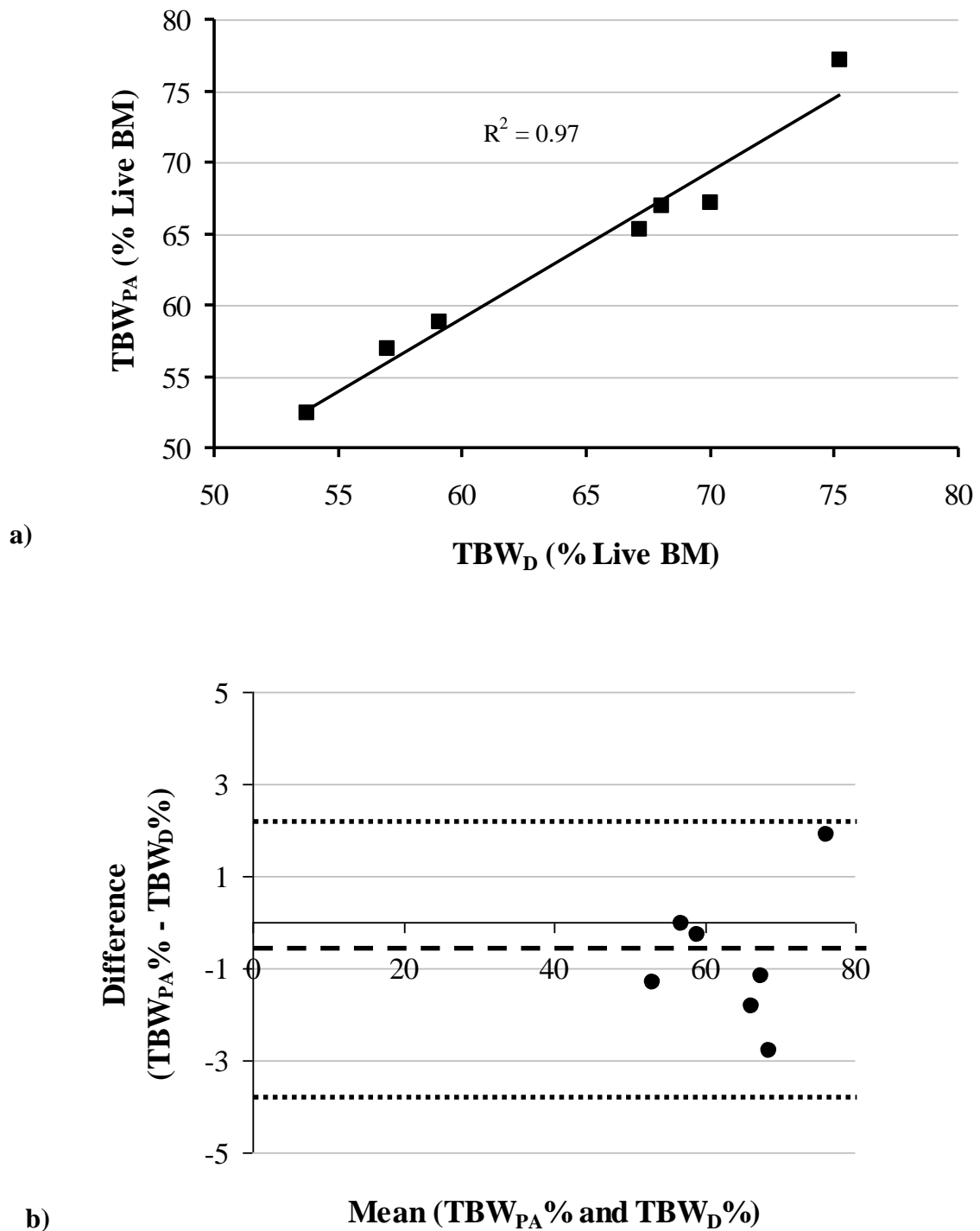
#### Footnote

Using a copy of the tabulated body condition scoring chart (Kohnke, 1992), for each pony, those terms which were deemed to apply to each pony were highlighted. The minimum and maximum numerical scores assigned to the chosen statements for each of the 6 body regions were then summed and averaged, providing overall minimum and maximum scores. These latter were subsequently averaged to the final body condition score. BCS values are therefore presented to two decimal places.



**Figure 5.1:** Total body water determined by proximate analysis (TBW<sub>PA</sub>) was inversely related to all measures of body ‘fat’: total body lipid (black diamonds), neutral body lipid (open diamonds), dissected white adipose tissue (open triangles) and D<sub>2</sub>O dilution-derived total body fat mass (black triangles), in seven mature pony mares which ranged in BCS (1.25 to 7/9).

D<sub>2</sub>O dilution-derived estimates of TBW<sub>D</sub> were strongly associated with TBW<sub>PA</sub>, both in terms of absolute mass ( $R^2 = 0.98$ ,  $P < 0.0001$ ) and when expressed as a percentage of BM ( $R^2 = 0.97$ ,  $P = 0.0001$ , Figure 5.2a, Table 5.2). If proximate analysis is accepted as the ‘gold standard’ technique for the measurement of TBW content, the strong agreement between TBW<sub>PA</sub> and TBW<sub>D</sub> demonstrated by Bland-Altman analysis, with all measurements lying within narrow ( $< \pm 5\%$ ) limits of agreement (LoA), suggested that the D<sub>2</sub>O dilution technique offered a valid alternative measurement method. It should be noted that TBW<sub>D</sub> over-estimated actual TBW (TBW<sub>PA</sub>) by 3.12kg (LoA: -8.80 to 2.56kg) or 0.79% Live BM (LoA: -3.75 to 2.17%, Figure 5.2b). Equations are presented to account for these differences (Table 5.2).



**Figure 5.2:** a) Linear regression and b) Bland-Altman analysis of total body water determined by both D<sub>2</sub>O dilution (TBW<sub>D</sub>) and proximate analysis (TBW<sub>PA</sub>). Data are expressed as percentages of live body mass for the seven, mature pony mares which ranged in BCS. The coefficient of determination ( $R^2$ ) is displayed above the regression line. The Bland-Altman plot (b) illustrates the mean difference between the two techniques (bias, dashed line) and the upper and lower limits of agreement (bias  $\pm$  1.96 standard deviations, dotted lines).

**Table 5.2:** Regression equations to enable prediction of either i & ii) proximate analysis-derived measures of total body water ( $TBW_{PA}$ ), iii & iv) total body lipid (TBL), v & vi) neutral body lipid (NBL) or vii & viii) dissection-derived white adipose tissue (WAT), from deuterium oxide dilution-derived measures of total body water ( $TBW_D$ , i & ii) and total body fat (TBFM, iii to viii). Equations are presented to allow the correction of data as either absolute mass (kg) or as a percentage of live body mass.

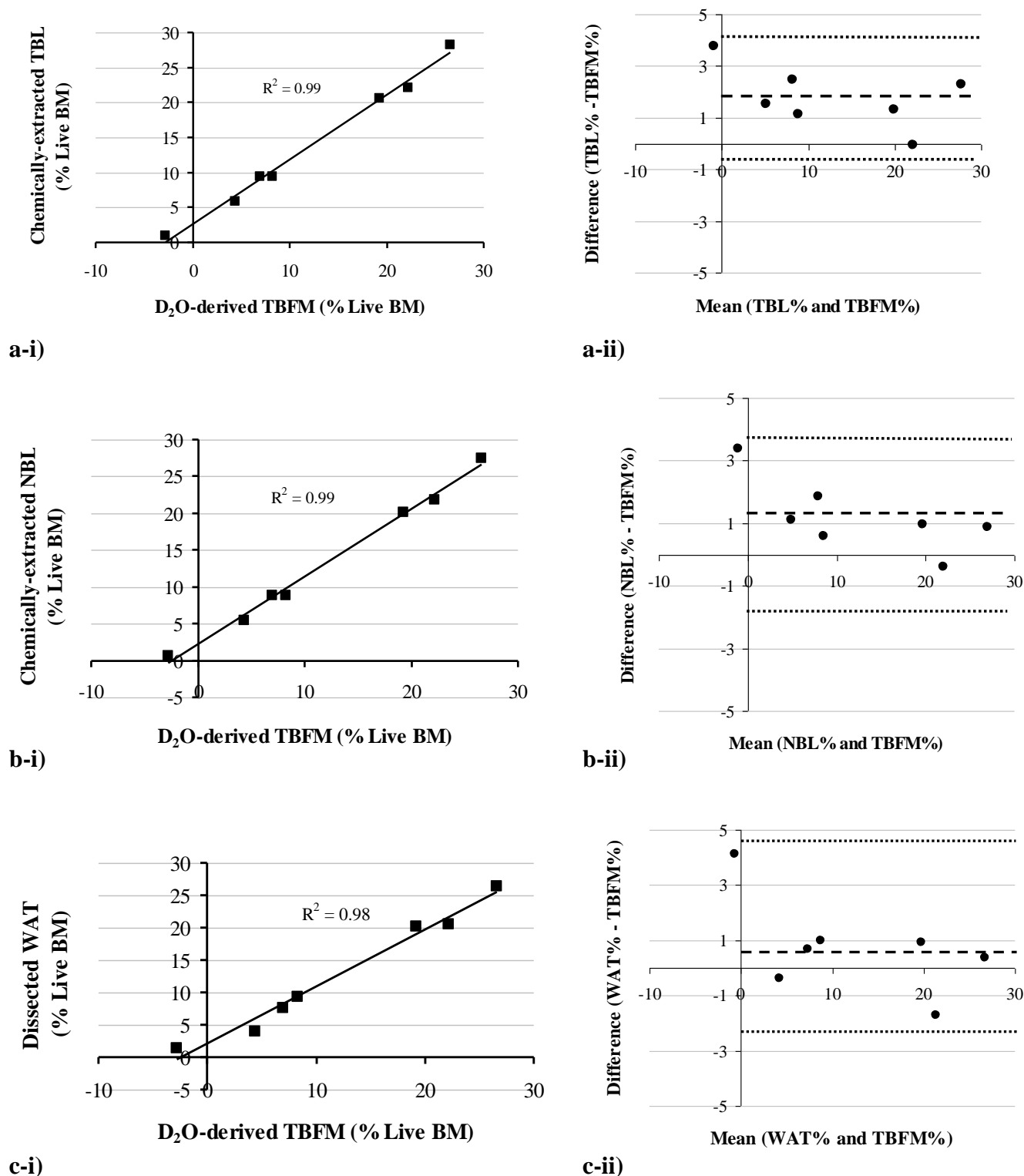
				95% confidence intervals	
Outcome	Regression Equation	$R^2$	P value	Slope	Intercept
Total Body Water	i) $TBW_{PA} = 5.8 + 0.93TBW_D$	0.98	0.0000	0.80 to 1.07	-13.44 to 25.06
	ii) $TBW_{PA}\% = -2.7 + 1.03TBW_D\%$	0.97	0.0001	0.81 to 1.25	-16.88 to 11.45
Total Body Lipid	iii) $TBL = 4.7 + 0.95TBFM$	0.99	0.0000	0.86 to 1.03	1.70 to 7.61
	iv) $TBL\% = 2.6 + 0.92TBFM\%$	0.99	0.0000	0.83 to 1.02	1.15 to 4.05
Neutral Body Lipid	v) $NBL = 3.9 + 0.94TBFM$	0.996	0.0000	0.86 to 1.01	1.22 to 6.52
	vi) $NBL\% = 2.2 + 0.92TBFM\%$	0.99	0.0000	0.83 to 1.00	0.84 to 3.52
Dissectable WAT	vii) $WAT = 3.4 + 0.91TBFM$	0.99	0.0000	0.80 to 1.03	-0.79 to 7.62
	viii) $WAT\% = 2.03 + 0.88TBFM\%$	0.98	0.0000	0.74 to 1.03	-0.19 to 4.26

D<sub>2</sub>O dilution-derived estimates of TBFM were highly correlated with chemically-extracted TBL and NBL mass ( $R^2 = 0.995$  and  $0.996$  respectively, both  $P < 0.0001$ ) and dissected WAT mass ( $R^2 = 0.99$ ,  $P < 0.0001$ ). This strong association between TBFM and other objective measures of body fat content was retained when each variable was expressed as a percentage of BM (TBL and NBL,  $R^2 = 0.99$ ,  $P < 0.0001$  for both; dissected WAT,  $R^2 = 0.98$ ,  $P < 0.0001$ , Figure 5.3 a-i, b-i and c-i). When Bland-Altman analyses compared D<sub>2</sub>O dilution-derived TBFM with TBL, NBL and WAT, good agreement was demonstrated in each case (Figure 5.3 a-ii, b-ii and c-ii). TBFM provided a small under-estimate of TBL (by 3.12kg, LoA: -1.27 to 7.51kg or 1.78% Live BM, LoA: -0.59 to 4.15% Live BM), NBL (by 2.10kg, LoA: -2.29 to 6.49kg or 1.20% Live BM, LoA: -1.09 to 3.49% Live BM) and dissected WAT (by 0.94kg, LoA: -5.63 to 7.51kg or 0.72% Live BM, LoA: -2.77 to 4.21% Live BM). Equations are offered to account for differences in methodologies (Table 5.2).

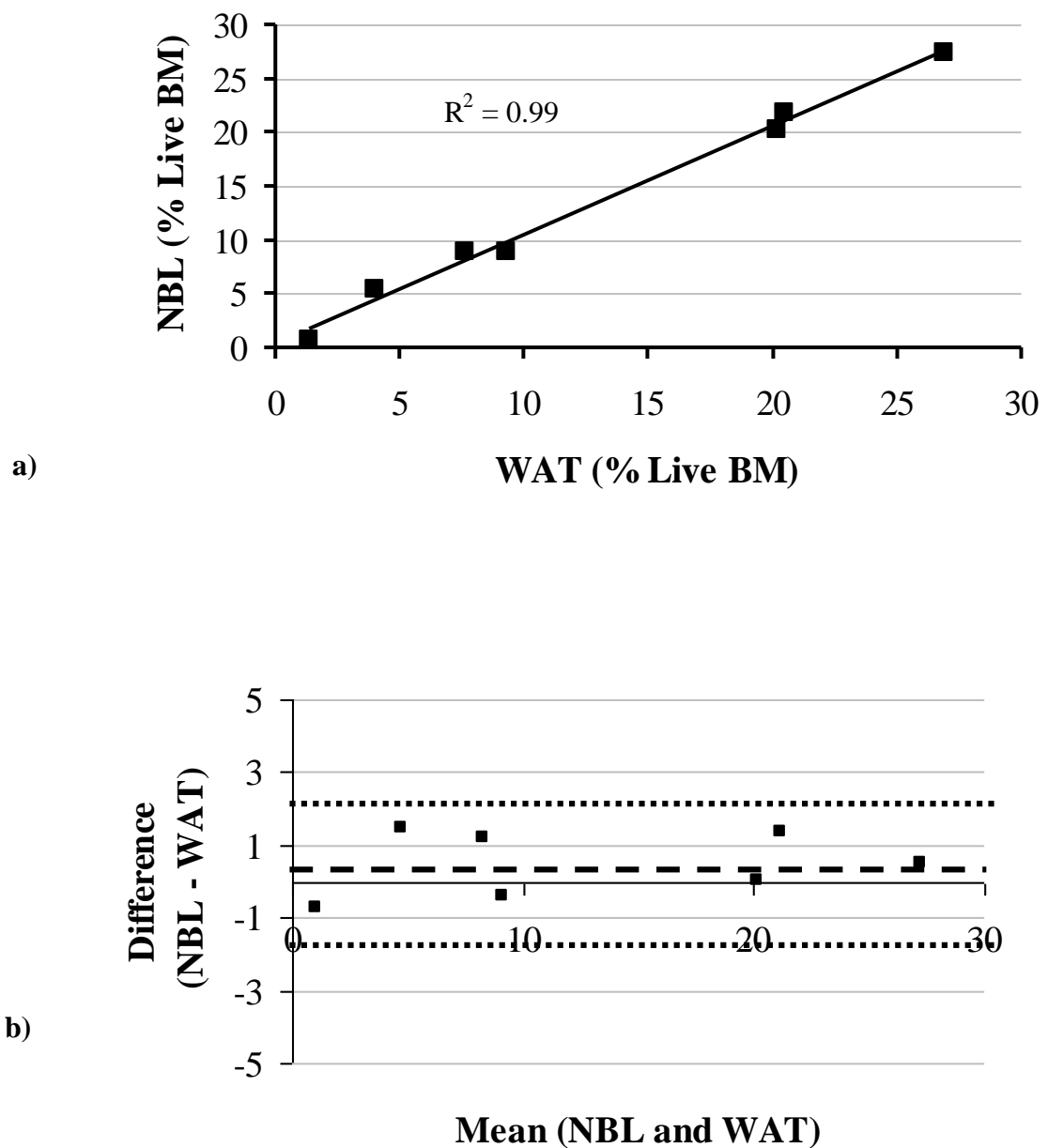
The total mass of WAT recorded following dissection, increased exponentially with BCS when data were normalised for differences in BM ( $R^2 = 0.95$ ) and ranged between 1.35 to 26.34% of BM<sub>PA</sub>. Although measures of dissected WAT differ qualitatively from measures of both NBL and TBL derived from proximate analyses, physical measures of WAT were strongly associated with chemical measures of NBL and TBL, whether these were expressed in terms of actual mass (both  $R^2 = 0.99$ ,  $P < 0.0001$ ) or as percentages of BM (both  $R^2 = 0.99$ ,  $P < 0.0001$ , Figure 5.4a). Exploration of the relationship using Bland-Altman analysis confirmed an excellent agreement between physical measures of WAT and both NBL and TBL (Figure 5.4b). However, minor adjustments would be required to correct for the slight under-estimates generated should WAT be used to indirectly determine actual body lipid



contents (TBL by 2.18kg, LoA: -1.58 to 5.94kg or 1.06% BM, LoA: -0.84 to 2.96% BM; NBL by 1.16kg, LoA: -2.41 to 4.73kg or 0.48% BM, LoA: -1.24 to 2.20% BM) (Figure 5.4b & Table 5.2). Structural/essential lipid (TBL minus NBL), formed only  $0.5 \pm 0.1\%$  of the remaining lean empty body mass (Live BM minus gut contents and NBL).



**Figure 5.3:** Linear regressions (i) and Bland-Altman agreement analyses (ii) of data describing values derived from values from seven, mature pony mares in a range of BCS. The percentage of total body fat derived from D<sub>2</sub>O dilution (TBFM) is regressed on **a)** the percentage total body lipid measured by proximate analysis (TBL); **b)** the percentage neutral body lipid measured by proximate analysis (NBL) and **c)** the percentage of white adipose tissue determined by dissection (WAT). The coefficients of determination ( $R^2$ ) are displayed above their respective regression lines of best fit. Bland-Altman plots illustrate the mean difference between the two techniques (bias, dashed line) and the upper and lower limits of agreement (bias  $\pm 1.96$  standard deviations, dotted lines).



**Figure 5.4:** a) Linear regression and b) Bland-Altman analysis of dissected white adipose tissue (WAT) and proximate analysis-derived neutral body lipid (NBL). Data are expressed as percentages of live body mass for the seven, mature pony mares which ranged in BCS. The coefficient of determination ( $R^2$ ) is displayed above the regression line. The Bland-Altman plot illustrates the mean difference between the two techniques (bias, dashed line) and the upper and lower limits of agreement (bias  $\pm$  1.96 standard deviations, dotted lines).

## 5.5 Discussion

This study reports the first definitive validation of the deuterium oxide dilution technique for the determination of both total body water and body fat against ‘gold standard’, dissection and chemical (proximate) analysis in *Equidae*.

Strong agreements were obtained between measurements of TBW pool size and body ‘fat’ generated by proximate analysis and following deuterium oxide dilution. The data support the use of the stable isotope method for the quantification of TBW and body ‘fat’, whether expressed as WAT, TBL or NBL in living animals. Equations are offered to account for subtle differences in absolute values between methods. That the absolute values generated by the D<sub>2</sub>O dilution and proximate analysis differed slightly was anticipated as each method evaluated different attributes of water and ‘fat’. Given the good association between calculated deuterium dilution space with body water content measured by desiccation and assuming an inverse relationship between TBW and body ‘fat’ content (Siri, 1956), good agreement between measures of body ‘fat’ and body lipid content might have been predicted. Less obvious was the useful observation that TBFM also offered a good estimate of dissectible (excluding intramuscular deposits) WAT, a tissue which inevitably includes non-adipose components. Only one animal (P7) had visible marbling of WAT between the fascicles of any dissected muscle. Conversely, WAT in the thinnest pony (P1) was notably gelatinous as a result of serous atrophy (Schoonderwoerd *et al.*, 1986). An ability to quantify WAT mass may be more readily appreciated by owners and clinicians alike and provides a strong case-management tool.

It is recognized that, irrespective of the strong agreements between methods and that this is the first attempt in any species to obtain concurrent data, the number of animals used in this trial is relatively small. While between-method agreements were extremely close given the complexity of the methods compared, the small sample size precludes the description of systematic errors within methods.

Measures of TBW generated for animals in the current study were comparable to those previously reported for horses and other species (Siri, 1956; Robb *et al.*, 1972; Sheng and Huggins, 1979). The good agreement (within  $\pm 5\%$ ) between measurement techniques in this trial may reflect the care taken to control, or account for, known sources of systematic error. Proximate analytical methods tend to underestimate TBW as a result of unaccounted water losses. Given the size of the animals and the oily nature of the tissues studied, the rapid handling, cold storage of polythene-wrapped tissues and the use of tissue lyophilisation over oven-drying were prioritised to minimise errors arising from evaporative water and volatile lipid loss (Johnson and Nagy, 2005).

Conversely, in the measurement of TBW by D<sub>2</sub>O dilution, factors influencing isotope distribution and loss have been associated with a tendency towards the over-estimation of TBW and an overall correction factor of 4% is commonly applied (Speakman *et al.*, 2001b). Previous studies using the D<sub>2</sub>O dilution technique reported over-estimations of TBW, commonly ranging from <1% to 6% but reaching 22.5% in one report (reviewed by Speakman *et al.*, 2001b). The 4% correction factor largely accounts for the isotopic exchange of deuterium with non-aqueous hydrogen in predominantly proteinaceous organic compounds (Culebras *et al.*, 1977; Schoeller,

2005). Application of this correction factor to current data greatly decreased between-method differences in TBW estimates ( $TBW_D$ , +0.79% cf.  $TBW_{PA}$ ). However, review of earlier studies in pigs and cattle would have suggested that application of the 4% correction factor would have resulted in a slight overcompensation (Houseman *et al.*, 1973; Wright and Russel, 1984).

The magnitude of the error in reported measures of  $TBW_D$  has also been related to the kinetics of  $D_2O$  distribution throughout the body water pool. Equilibration rates for administered deuterium throughout the digesta water have been questioned (Sheng and Huggins, 1979; Arnold and Trenkle, 1986; Speakman *et al.*, 2001b). Unlike omnivores in which digesta water may account for <5% of TBW (Cizek, 1954; Sheng and Huggins, 1979), in herbivores, this reserve can be relatively substantial (cattle ~10.6% of TBW, Arnold *et al.*, 1985; rabbits ~12%, Gotch *et al.*, 1957; horses 8-18 %, Meyer, 1996). The equilibration of deuterium in equine digesta might be inferred to occur between 3 and 5h after intravenous  $D_2O$  administration, when plasma deuterium enrichments became relatively constant (Fuller *et al.*, 2004). The 4h period allowed for equilibration in this study acknowledged the findings of Fuller *et al.*, (2004) who used ponies of the same breed.

In the current study, digesta accounted for ~11% of BM and 12-23% of TBW, which was comparable with previous reports (Robb *et al.*, 1972; Webb and Weaver, 1979; Meyer, 1996). The water content of digesta (~90%) was also similar to the 85-90% range reported for horses (Meyer, 1996) and ruminants (Cowan *et al.*, 1979; Andrew *et al.*, 1995). Differences in digesta hydration between animals, which might have influenced isotope equilibration rates (Arnold and Trenkle, 1986) were

minimised by establishing the ponies (5/7) on a common, forage-based diet. Although digesta hydration was constant between ponies, gut fill and total digesta water varied inversely with BCS. This association has also been demonstrated in cattle and presumably reflects the decreased voluntary food intakes recorded in fatter representatives of each species (Bines *et al.*, 1969; Dugdale *et al.*, 2010). The relatively large digesta water in the leanest pony (P1) may have been, at least partly, causal to the negative value of TBFM in this animal (Andrew *et al.*, 1995).

The kinetics of deuterium distribution are also influenced by the rate of body water turnover (Edelman, 1952; Moore *et al.*, 1968; Speakman *et al.*, 2001b). For the purposes of TBW<sub>D</sub> estimation, a more stable (slowly decaying) phase of relatively constant plasma deuterium enrichment was promoted by withholding access to food and water immediately before and during the equilibration period (Speakman *et al.*, 2001b). Measurement error was minimised by using the preferred technique of gas isotope ratio mass spectrometry, where measurement CVs were consistently within 0.5ppm (Burkholder and Thatcher, 1998; Speakman *et al.*, 2001b).

In this study, plasma was sampled to determine equilibrated deuterium enrichments after 4 hours (Fuller *et al.*, 2004). Any delay in sample collection was considered likely to introduce errors due to direct and indirect mechanisms which could contribute to absolute or relative isotope loss. These mechanisms include: water elimination by sensible and insensible routes; water elution by continued intake and metabolic water synthesis; isotopic fractionation; and incorporation of tracer into newly synthesised molecules (Moore *et al.*, 1968; Speakman *et al.*, 2001b). These mechanisms may be most significant as potential sources of error in growing,

breeding, lactating or exercising animals in which metabolic rates are increased (Moore *et al.*, 1968; Odwongo *et al.*, 1984; Wong *et al.*, 1998; Speakman *et al.*, 2001b; Schoeller, 2005). Deuterium lost as methane excreted from the digestive tract was considered a negligible source of error in the estimation of TBW<sub>D</sub> (Fuller *et al.*, (2004).

Species-specific physiological differences also require consideration as potential sources of deuterium loss. Deuterium losses in urine or faeces have not been reported for *Equidae*, were discounted in dogs but considered an important source of error in pig studies (Houseman *et al.*, 1973; Andrews *et al.*, 1997; Burkholder and Thatcher, 1998; Fuller *et al.*, 2004). Faecal and urinary losses from ponies in the current study were minimized by withholding food and water (Odwongo *et al.*, 1984). Intravenous administration of D<sub>2</sub>O has been suggested to cause marked respiratory losses of isotope due to the first pass of the enriched bolus through the pulmonary vascular bed (Burkholder and Thatcher, 1998). Animals in the current study were housed and maintained within their thermoneutral zone to minimise respiratory deuterium loss. In the dog, for which the insensible loss of respiratory water provides an important thermoregulatory function, respiratory deuterium losses were considered to contribute to the over-estimation of TBW<sub>D</sub> (Burkholder and Thatcher, 1998). This error in the dogs, which might have been due to either loss or gain of water via the respiratory tract, may have been exacerbated by the rebreathing system used to provide oxygen during maintenance of general anaesthesia (Nagy and Costa, 1980).

The inverse relationship between body water and lipid content is well documented and supported by data in this study (Siri, 1956). It has been suggested



that body composition could also influence the kinetics of deuterium distribution and longer equilibration times have been suggested for obese subjects (Schoeller *et al.*, 1980; Wells and Fewtrell, 2006).

In the current study, total body lipid content, determined following chemical extraction, ranged from 1 to 28% of BM<sub>PA</sub> and this range was closely reflected by body contents of dissected WAT and values of TBFM derived following deuterium dilution. Similar body WAT contents have been reported in a variety of horse and pony breeds, although associations with BCS were not recorded (5.6% of live BM (<1 to >11%), Webb and Weaver, 1979).

The calculation of TBFM from TBW is dependent on the assumption that TBW is entirely associated with 'lean' body tissues which have an overall constant hydration factor of 73.2% (Pace and Rathbun, 1945). Although this hydration factor has been the subject of much debate, it has generally been supported for mature mammals (Sheng and Huggins, 1979; Wang *et al.*, 1999; Schoeller, 2005). However, small variations in lean tissue hydration will bias TBFM values calculated from TBW. This may be an important consideration for animals at the extremes of the BCS scale (Sheng and Huggins, 1979; Wang *et al.*, 1999) and may have contributed to the negative value for TBFM recorded in the leanest animal (P1, Sheng and Huggins, 1979; Wright and Russel, 1984).

## **5.6 Conclusion**

This study provides the first validation of the D<sub>2</sub>O dilution method for the minimally-invasive, accurate, repeatable and objective measurement of body water

and fat in living *Equidae*. Controlled application of the technique in accordance with suggested protocols should facilitate the clinical assessment of equine body composition and promote useful epidemiological exploration of the associations between body composition, health and performance.

## 5.7 References

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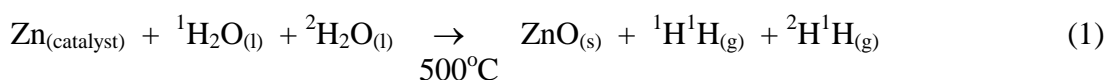
# **Supplementary Information 2**

## SI 2.1 Measurement of deuterium enrichment in plasma samples

### SI 2.1.1 Expanded Materials and Methods

#### *A. Overview of zinc reduction method*

1) Basic redox reaction:-



Deuterium gas, as  ${}^2\text{H}_2$  is rarely formed, unless the quantity of deuterium oxide present approaches 100%.

2) Mass spectrometry:-

Gaseous hydrogen species are ionised within the mass spectrometer as follows:-



The ratio of these ions is then analysed by gas isotope ratio mass spectrometry (gIRMS) (see below).

#### *B. Reduction vessels*

Specialised glass (fused quartz) reduction vessels (Louwers Hapert Glastechniek BV, Nijverheidsweg, The Netherlands), capable of withstanding evacuation (down to  $\sim 10^{-4}$  mbar during preparation and  $\sim 10^{-9}$  mbar during mass spectrometry) and a range of temperatures (liquid nitrogen [ $-196^\circ\text{C}$ ] to  $+500^\circ\text{C}$ ) were used. Each vessel was supplied with its own ‘stopper’ which screwed onto the upright open end of the reduction vessel and operated an integral stop-cock which drove a fused quartz piston up and down inside the top part of the tube to either include or exclude the side-arm from the distal part of the tube body. Each piston was equipped with three rubber ‘O’ rings which were lubricated with silicone grease (Silicone grease M494; Ambersil Ltd. Bridgwater, UK) to ensure an air-tight seal.

#### *C. Preparation of samples for analysis by gIRMS*

Clean, oven-dried vessels were prepared in batches of 7 as follows. Two hundred ( $\pm 10$ ) milligrams of Hayes zinc reagent (Biogeochemical Laboratories, Bloomington, Indiana, USA), which had been stored under vacuum to prevent oxidation, were weighed on a calibrated and regularly performance-checked precision balance (Sartorius Basic; Sartorius AG, Goettingen, Germany), and transferred to the bottom of each reduction vessel via a glass funnel. The stopper was then inserted and the vessel attached to a vacuum manifold along with 6 others and evacuated to  $\sim 5 \times 10^{-2}$  mbar. While this vacuum was maintained, the 7 vessels were heated to  $\sim 450^\circ\text{C}$  using a hot air gun (Steinel HL1605S Thermo control speed; Steinel, Bloomington,



Minnesota, USA) for 1.25min each to ensure no moisture remained within them. Each vessel was subsequently evacuated to  $<10^{-4}$  mbar and allowed to cool.

The manifold was then isolated from the vacuum system and filled with dry nitrogen gas, under slight positive pressure ( $\sim 1-1.5 \times 10^3$  mbar), flowing into the manifold at 4-5L/min. For each vessel in turn, the stopper was removed and 7.5uL of sample was introduced into the vessel using a slim micro-pipette and tip (Socorex micropipette and Qualitips™; Socorex Isba S.A., Ecublens, Switzerland); the sample being deposited on the inside wall of the vessel sufficiently proximal to the zinc to prevent mixing with the zinc and also to ensure no subsequent direct contact with the heating block so as to prevent sample combustion. The stopper was then replaced. Once all 7 vessels had had their respective samples added, they were immersed in liquid nitrogen to a level above the deposited samples for 6min in order to freeze the samples (to prevent any isotopic fractionation). During this time, the tubes were re-evacuated to  $<10^{-4}$  mbar. Batches of 7 prepared sample vessels were then stored on crushed ice until a total of 21 reaction vessels had been prepared.

Batches of 21 vessels were then placed in a specially designed heating block (Techné Dri-Block DB-4; Techné, part of Barlow Scientific Ltd., Staffordshire, UK), at 500°C ( $\pm 5^\circ\text{C}$ ) [temperature monitored with a thermocouple probe; FLUKE 52 K/J; Fluke Corporation, Everett, Washington, USA] for 30min to catalyse the reaction between the sample and the zinc. Once cool, the  $^2\text{H}^1\text{H}$  and  $^1\text{H}^1\text{H}$  gases present in the vessels were analysed by a mass spectrometer fitted with split flight tube and HD collector and 20-port sample manifold (SIRA 10, VG Isotech, Cheshire, UK). In the mass spectrometer, the gaseous hydrogen species were ionised by electron impact as follows:-



The  $^1\text{H}^1\text{H}^+$  ion has a mass:charge (m/z) ratio of 2, whereas the  $^2\text{H}^1\text{H}^+$  species has an m/z of 3. The ratio of these two species (m/z 3: m/z 2) was then determined by the mass spectrometer. However, a parasitic ion,  $^1\text{H}_3^+$ , may be produced during the ionisation process in the mass spectrometer as follows:-



$^1\text{H}_3^+$  also has an m/z of 3. The production of this ion is dependent upon the partial pressures of  $^1\text{H}^1\text{H}^+$  and  $^1\text{H}_2$  in the ion source of the mass spectrometer. Standard corrections for the presence of this ion were therefore applied to the measured m/z 3: m/z 2 ratios by the instrument software during the measurement process.

Three different internal standard reference ‘water’ samples were analysed alongside each batch of samples. These were Aberdeen tap water (with deuterium enrichment of  $\sim 150$ ppm), and a standard with deuterium excess of  $\sim 300$ ppm (i.e.  $\sim 450$ ppm in total) and, to ensure reproducibility of method, a quality control standard with  $\sim 280$ ppm deuterium enrichment. The first two ‘waters’ were included in order to generate a standard (calibration) plot. The isotopic composition of these reference waters had previously been normalised against the international standards V-SMOW and SLAP according to Gonfiantini (1978).

The reference hydrogen gas was prepared from Aberdeen tap water. The deuterium enrichments of the samples were measured in  $\delta^2\text{H}_2$  units (‰), relative to Aberdeen tap water:-

$$\delta\text{‰} = \frac{\text{Ratio sample-1}}{\text{Ratio working standard}} \times 1000 \quad (5)$$

where, ratio = m/z 3 : m/z 2 and the working standard is Aberdeen tap water. The  $\delta(\text{‰})$  values were subsequently converted into parts per million using established values for the reference standards as already presented in the Materials and Methods (Chapter 5).

# Chapter 6

**Can body condition  
scoring be used to  
determine body fat in  
*Equidae*?**

## 6.1 Summary

Body condition scoring systems have become commonly used for the assessment of body fat although were originally intended to evaluate the superficial flesh (both muscle and fat) covering of the skeletal frame. This study therefore aimed to investigate the relationship between body fat content (derived by deuterium oxide dilution) and body condition score (BCS) in a mixed group of mature horses and ponies studied throughout the year.

For 48 animals, body fat (derived by the deuterium oxide dilution technique) and BCS (subjectively appraised according to a previously described system on a scale of 1 [emaciated] to 9 [obese]), were collected concurrently as part of a larger data set maintained during various feeding trials. Data from 45 animals were included in the final analyses. Linear regression modelling was used to determine the relationship between body fat percentage and BCS.

The 48 mature animals were from a range of breeds, although Welsh Mountain ponies predominated ( $n = 37$ ), and sexes, with median (range) age 12yr (5-24), body mass 257kg (160-764) and withers height 116cm (85-169). Body fat percentage was non-linearly related to BCS such that  $BCS^2$  had the best linear association with body fat content. Due to wider residual variation, a BCS value of 7/9 was determined as a cut off value. Values of  $BCS < 7$  were found to be good predictors of body fat content ( $R^2 = 0.86$ ;  $P < 0.0001$ ), but values  $\geq 7$  were poor predictors of body fat content ( $R^2 = 0.10$ ;  $P = 0.13$ ).

This study highlights the subjective nature of BCS and warns of its poor ability to predict fatness once an animal is already fat. BCS descriptors may need refining in order to improve BCS systems to sub-categorise fat animals, although once fat ( $\text{BCS} \geq 7$ ), these animals are likely to be at increased risk of obesity-related disease.

## 6.2 Introduction

Body Condition Scoring (BCS) systems were originally developed for the food animal industry to evaluate the superficial ‘flesh’ (both muscle and fat) covering of animals in order to facilitate their management and improve productivity (Jeffries, 1961). As such, these systems were not originally intended for the evaluation of body fat. Nevertheless, with the current increase in companion animal obesity (German, 2006; Sillence *et al.*, 2006), BCS systems have become accepted as measures and monitors of body ‘fatness’, at least in dogs and cats (Laflamme, 1997a & b). Recent work in ponies, however, has confirmed earlier work in horses which suggests caution in applying equine BCS systems to measure/monitor ‘fatness’ in *Equidae*, due to the non-linear association between body fat content and BCS (Martin-Rosset *et al.*, 2008; Dugdale *et al.*, In Press a).

Additionally, the development of many BCS systems, even those used in the farm animal industry, has been criticised for a tendency to focus on observer error in score application (due to poor or inadequate detail or a misunderstanding of the descriptors), whilst omitting to ensure rigorous validation against more objective measures of body flesh or, indeed, fat (Charette *et al.*, 1996). With this in mind, a widely used, equine body condition scoring was recently validated as an accurate predictor of ‘flesh’ (total soft tissue content) against the gold standard method of carcass dissection (Henneke *et al.*, 1983; Kohnke, 1992; Dugdale *et al.*, In Press a).

The body fat component of ‘flesh’ is highly variable. Given the previously described, non-linear association between equine BCS and body fat content, the ability of BCS systems to predict body fat content warranted further investigation in a

larger number of animals. Validation of the deuterium oxide (D<sub>2</sub>O) dilution method as an objective, yet non-destructive, method for determination of equine body fat content has enabled expansion of the data set across a larger population with the inclusion of animals of mixed breed and sex (Dugdale *et al.*, In Press b). The present study investigated the relationship between body fat content (derived by D<sub>2</sub>O dilution), and BCS in a mixed group of 48 horses and ponies studied throughout the year.

### 6.3 Materials and Methods

Data from 48 horses and ponies, predominantly (37/48) Welsh Mountain ponies, recruited onto various feeding trials at the University of Liverpool (Dugdale *et al.*, 2010 & In Press a & under review c; Curtis *et al.*, 2010) were available for analysis. For each animal, age, sex, breed and body mass were recorded and BCS was subjectively appraised, using Kohnke's modification (1992) of the system originally described by Henneke *et al.*, 1983 (BCS 1 [very poor] to 9 [extremely fat]; Table 6.1), by the same observer on each occasion (AD). On the same day, total body fat mass (TBFM), was calculated from D<sub>2</sub>O dilution-derived measurement of total body water, as described previously (Dugdale *et al.*, In Press b). Body fat percentage was calculated from TBFM and the body mass.

During application of any BCS system, a different score may result depending upon whether the animal is scored 'downwards' from the highest value or 'upwards' from the lowest value (AD; personal observation). Body condition score was therefore determined for each animal after initially highlighting all terms judged to be applicable to that individual in the BCS recording sheet (Table 6.1). Consequently, a range of scores was available for each body region appraised which, when summed and averaged in accordance with Kohnke's instructions, provided overall minimum ( $K_{low}$ ), maximum ( $K_{hi}$ ), and mean ( $K_{ave}$ ) scores for each individual. These three alternative BCS values were used in subsequent statistical analyses.



**Table 6.1:** Body condition scoring chart, from Kohnke, 1992.

Condition score	General condition	Neck	Withers	Loin	Tailhead	Ribs	Shoulder
<b>1</b>	<b>Very poor</b>	Individual bone structure visible.	Bones easily visible. No fat.	Spine bones visible. Ends feel pointed.	Tailhead and hip bones very visible.	Ribs very visible and skin furrows between ribs.	Bone structure very visible.
		Animal extremely emaciated: no fatty tissue can be felt.					
<b>2</b>	<b>Very thin</b>	Bones just visible. Animal emaciated.	Withers obvious. Very minimal fat covering.	Slight fat covering over vertical and flat spine projections. Ends feel rounded.	Tailhead, hip bones obvious.	Ribs prominent, slight depression between ribs.	Bone structure can be outlined.
<b>3</b>	<b>Thin</b>	Thin, flat muscle covering.	Withers accentuated with some fat cover.	Fat build-up halfway on vertical spines but easily discernible. Flat spinal bones not felt.	Tailhead prominent. Hip bones appear rounded but visible. Pin bones covered.	Slight fat cover over ribs. Rib outline obvious.	Shoulder accentuated. Some fat.
<b>4</b>	<b>Moderately thin</b>	Neck some fat, not obviously thin.	Withers not obviously thin, smooth edges.	Slight ridge along back.	Fat can be felt.	Faint outline visible.	Shoulder not obviously thin.
<b>5</b>	<b>Moderate</b>	Neck blends smoothly into body.	Withers rounded over top.	Back level.	Fat around tailhead beginning to feel spongy.	Ribs cannot be seen but can be easily felt.	Shoulder blends smoothly into body.
<b>6</b>	<b>Moderately fleshy</b>	Fat can be felt.	Fat can be felt.	May have slight inward crease.	Fat around tailhead feels soft.	Fat over ribs feels spongy.	Fat layer can be felt.
<b>7</b>	<b>Fleshy</b>	Visible fat deposits along neck.	Fat covering withers is firm.	May have slight inward crease down back.	Fat around tailhead is soft and rounded off.	Individual ribs can still be felt.	Fat build-up behind shoulder.
<b>8</b>	<b>Fat</b>	Noticeable thickening of neck.	Area along withers filled with fat.	Crease down back evident.	Tailhead fat very soft and flabby.	Difficult to feel ribs.	Area behind shoulder filled in flush with body.
		Fat deposited along inner buttocks					
<b>9</b>	<b>Extremely fat</b>	Bulging fat.	Bulging fat.	Obvious deep crease down back.	Bulging fat around tailhead.	Patchy fat over ribs.	Bulging fat.
		Fat along inner buttocks may rub together. Flank filled in flush.					
<b>Score</b>							

Scoring method: each area to be assessed individually, the scores totalled, then averaged to give the Condition Score

### *Statistical analyses*

Raw data were initially entered into a spreadsheet (Excel, Microsoft Office Professional Edition 2003; Microsoft Corp., Washington, U.S.A.) and subsequent statistical evaluation was performed using STATA 10 (Stata/IC 10.1: Stata Corp., Texas, U.S.A.).

The relationship between body fat percentage and BCS was investigated using linear regression modelling with body fat percentage as the outcome variable. Prior to modelling, the distributions of the three raw BCS values, namely  $K_{hi}$ ,  $K_{ave}$  and  $K_{low}$  and their various transformations (square, cubic, square root, log, inverse square root, inverse, inverse square and inverse cubic) were visually appraised using the *gladder* command in Stata according to Tukey's "Ladder of Powers" (1977). This was performed in order to select the optimal transformation prior to modelling. The optimal transformations were: none for  $K_{low}$  and squared for both  $K_{ave}$  and  $K_{hi}$ .

The association between BCS and body fat percentage as measured by  $D_2O$  dilution was investigated with linear regression with BCS as the explanatory variable and body fat percentage as the outcome variable. Separate linear regression models were fitted for each BCS after transformation if required i.e.  $K_{low}$ ,  $K_{ave}^2$ ,  $K_{hi}^2$ . The three models were compared using the Bayesian Information Criterion (BIC) (Long, 1996). For a model  $M_k$  with deviance  $D(M_k)$  the BIC is estimated as:  $BIC_k = D(M_k) - df_k^* \ln N$  where  $df_k^*$  is the degrees of freedom associated with the deviance and  $N$  is the sample size. The more negative the  $BIC_k$  the better the model fit, with an absolute difference in BIC between two models greater than 6 offering strong support for the model with the smallest BIC. This suggested that the linear regression model with

$K_{ave}^2$  as the explanatory variable had the best fit and was therefore used for subsequent analyses.

Model fit was further investigated by plotting actual values against fitted (predicted) values and by plotting the residuals and standardised residuals against fitted values. Standardised residuals were plotted against raw BCS ( $K_{ave}$ ) in order to appreciate any systematic variation across the range of BCS values.

The apparent “flaring” of standardised residuals at higher BCS values ( $\geq 7/9$ ) suggested poorer model fit at these high values. To further investigate this, the dataset was therefore split into “non-obese” and “obese” animals with the cut-off being at BCS 7. Separate regression models were subsequently fitted for each group of animals with body fat percentage as the outcome variable.

## 6.4 Results

Forty eight horses and ponies from a range of body condition scores (1.25 to 8.92/9) and of mixed breeds, ages and sexes were recruited (Tables 6.2 and 6.3). Data from 3/48 animals were excluded from subsequent analyses as a result of sample handling error ( $n = 1$ , Welsh Mountain pony mare, 5yr old, BCS 5/9) or negative body fat percentages (two thin animals; both Welsh Mountain pony mares, one 5yr old, BCS 2.67/9; one 13yr old, BCS 1.25/9).

**Table 6.2:** Descriptive statistics for age, body mass and withers height of the 48 horses and ponies studied.

Attribute	Number of animals	Mean	Median	Range
Age (yr)	48	11.2	12	5 – 24
Body Mass (kg)	48	305.0	256.5	159.5 - 764
Withers Height (cm)	48	119.9	115.5	85 - 169

**Table 6.3:** Breed and sex distribution of the 48 horses and ponies studied.

Breed	Male	Female	Total number
Shetland	2	0	2
Welsh Mountain Pony (Section A)	0	37	37
Welsh Pony (Section B)	0	1	1
Cob	3	3	6
Thoroughbred X	0	1	1
Warmblood	1	0	1

The best statistical model predicted body fat percentage according to  $K_{ave}^2$  (Table 6.4). For this final model, plots of both the residuals and of the standardised residuals against predicted (fitted) values indicated slight deviation from normality for higher predicted body fat percentages (Figure 6.1). A quantile-quantile (Q-Q) plot, comparing the data against theoretically expected normal values, displayed some deviation from the straight line, also indicating imperfect normality of the data (Figure 6.2). Given the relatively small sample size, it was considered that the data were sufficiently normally distributed to further investigate the model when the data were divided between high and low BCS scores (high and low body fat percentages).

When actual BCS ( $K_{ave}$ ) was plotted against the standardised residuals from the final model, flaring (increasing variation in the residuals) was noted at BCS values  $\geq 7$  (Figure 6.3), suggesting poorer model fit at these BCS values. To investigate differences in performance of the BCS system across its range, data for ‘non-obese’ animals ( $BCS < 7$ ) and ‘obese’ animals ( $BCS \geq 7$ ) were separated and linear regression models were independently fitted for the resultant subgroups: non-obese\_ $K_{ave}^2$  ( $n = 21$  [3 excluded animals]) and obese\_ $K_{ave}^2$  ( $n = 24$ ) (Table 6.4).

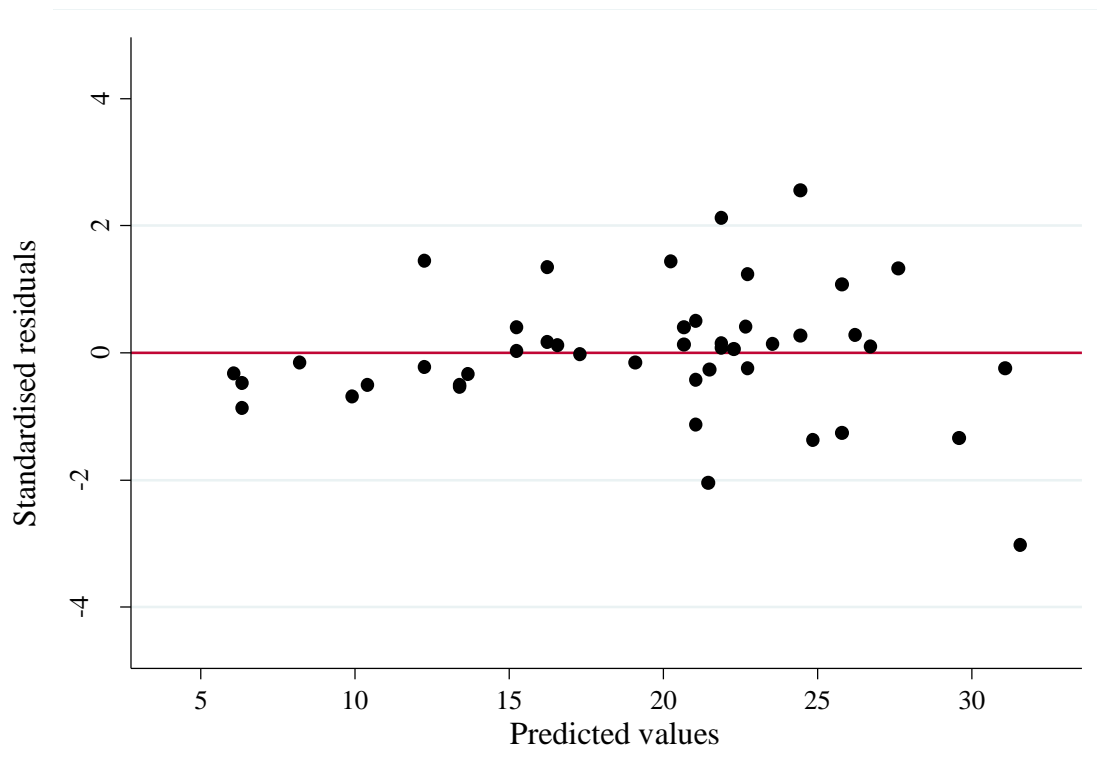
Scatterplots of the standardised residuals against predicted values for both these new models showed reasonable normality of data (Figure 6.4). Q-Q plots demonstrated a greater apparent normality for the ‘obese’ animals most probably as a result of the relatively restricted range of possible BCS scores for these animals (Figure 6.5).

Linear regression models suggested better association between  $K_{ave}^2$  and body fat percentage when  $K_{ave} < 7$  ( $R^2 = 0.86$ ;  $P < 0.001$ ,  $R^2 = 0.86$ ) [non-obese animals] compared with when  $K_{ave}$  was 7 or greater ( $R^2 = 0.10$ ;  $P$  not significant;  $R^2 = 0.10$ ) [obese animals] (Table 6.4).

The three models are represented within one figure for clarity, where the wider spread of data points at higher  $K_{ave}$  values coincides with a much reduced ability of  $K_{ave}$  body condition scores to predict body fat percentage (Figure 6.6).

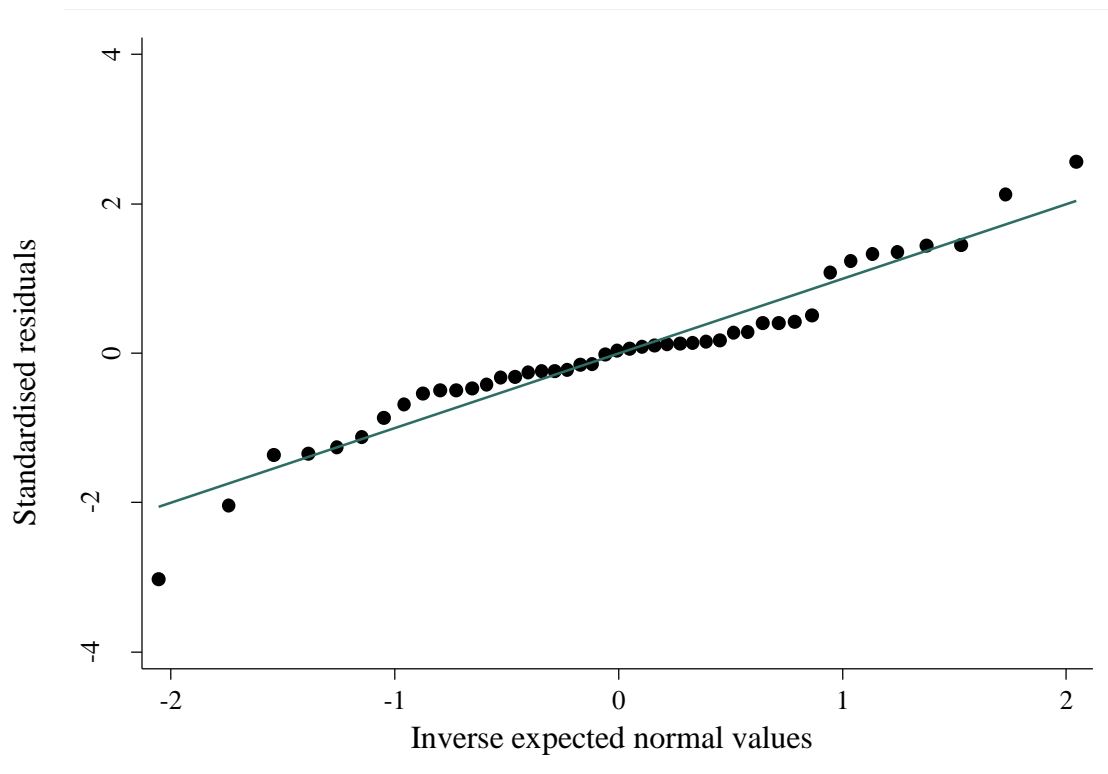
**Table 6.4:** Linear regression models for 1) the association between BCS ( $K_{ave}^2$ ) and body fat percentage measured by D<sub>2</sub>O dilution for all animals (n = 45); 2) the association between BCS (non-obese\_ $K_{ave}^2$ ) and body fat percentage for non-obese animals ( $K_{ave} \leq 7$ ; n = 21); and 3) the association between BCS (obese\_ $K_{ave}^2$ ) and body fat percentage for obese animals ( $K_{ave} > 7$ ; n = 24).

Model	Variable	Coefficient	Standard error	t	P	95% confidence intervals	Number of observations	R <sup>2</sup>
<b>1</b>	$K_{ave}^2$	0.34	0.04	9.84	< 0.0001	0.27 to 0.41	45	0.69
	Constant	4.20	1.68	2.50	0.016	0.81 to 7.59		
<b>2</b>	non-obese_ $K_{ave}^2$	0.46	0.04	10.97	<0.0001	0.37 to 0.54	21	0.86
	Constant	1.24	1.29	0.97	0.346	-1.45 to 3.93		
<b>3</b>	obese_ $K_{ave}^2$	0.19	0.12	1.56	0.132	-0.06 to 0.44	24	0.10
	Constant	13.03	7.21	1.81	0.084	-1.91 to 28.00		

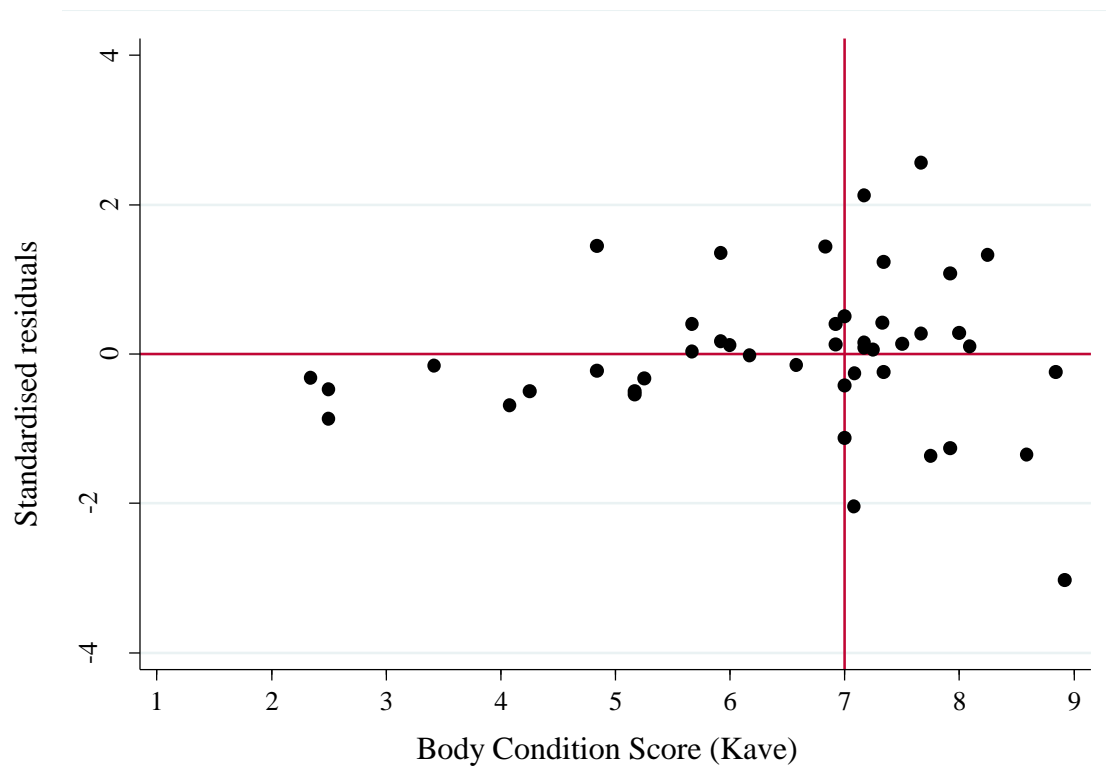


**Figure 6.1:** Scatterplot of standardised residuals against predicted values from the final linear regression model for all animals ( $n = 45$ ). Some deviation from normality is apparent for higher values of predicted total body fat percentage ( $>20\%$ ) which show a larger variance about the mean of zero.

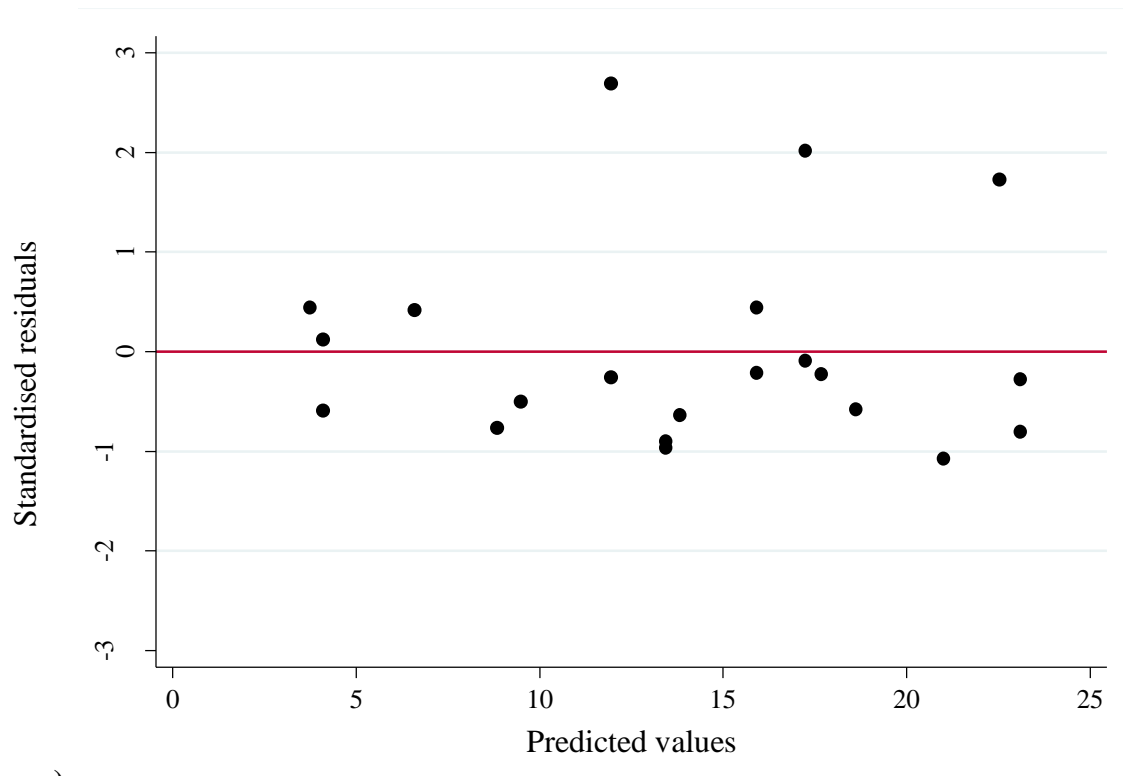




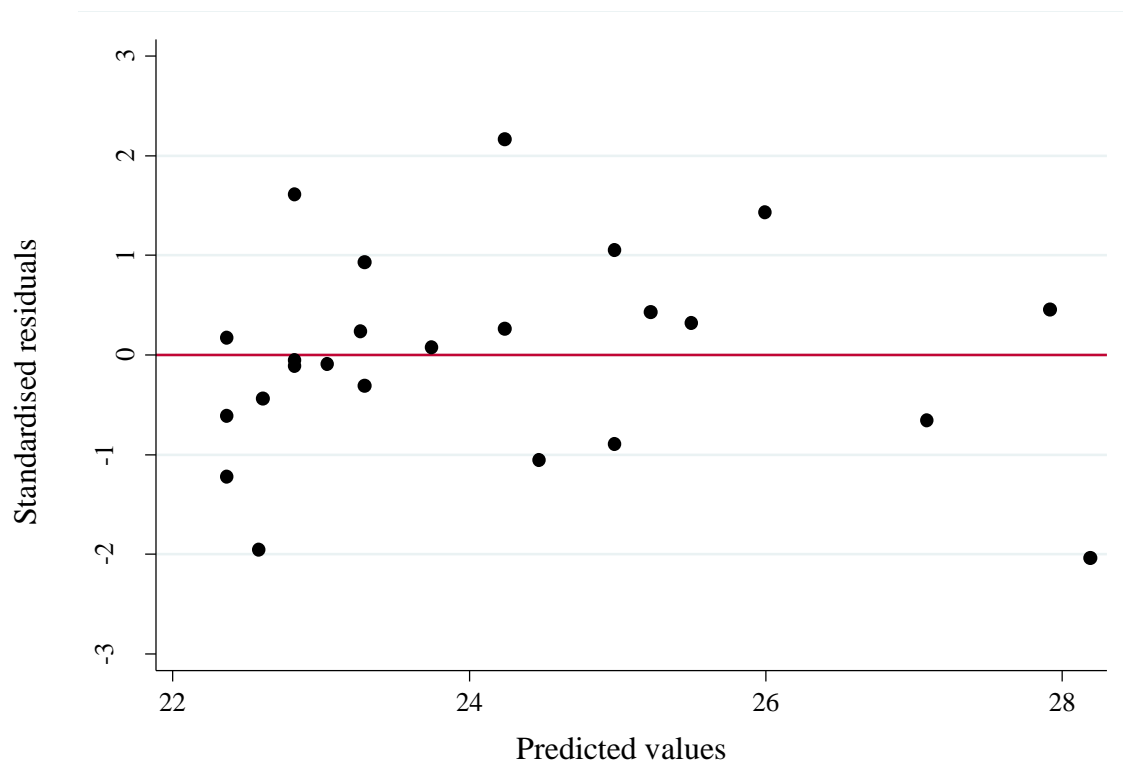
**Figure 6.2:** A quantile-quantile plot of the standardised residuals from the final linear regression model for all animals ( $n = 45$ ) against the theoretically expected, inverse normal values. Imperfect normality is suggested by deviation of data points from the line.



**Figure 6.3:** Scatterplot of actual body condition scores ( $K_{ave}$ ) against the standardised residuals from the final model for all animals ( $n = 45$ ). Flaring of data points is apparent for  $K_{ave}$  values greater than  $7/9$ .

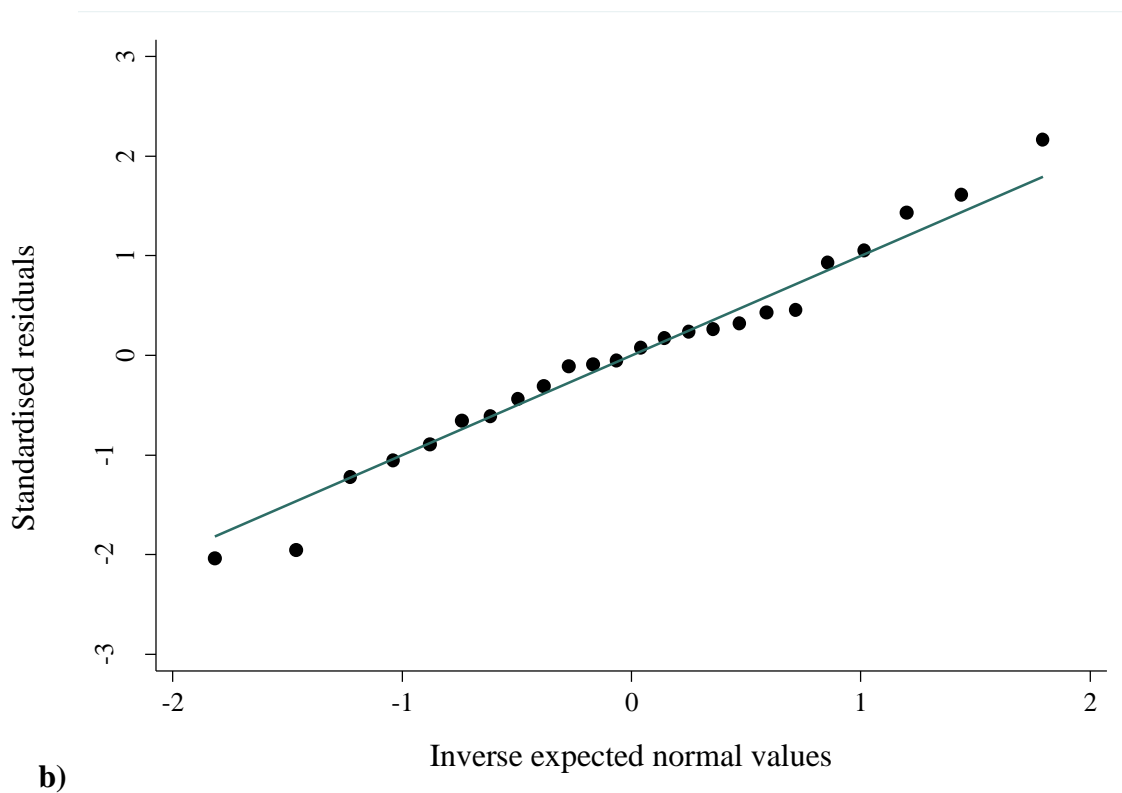
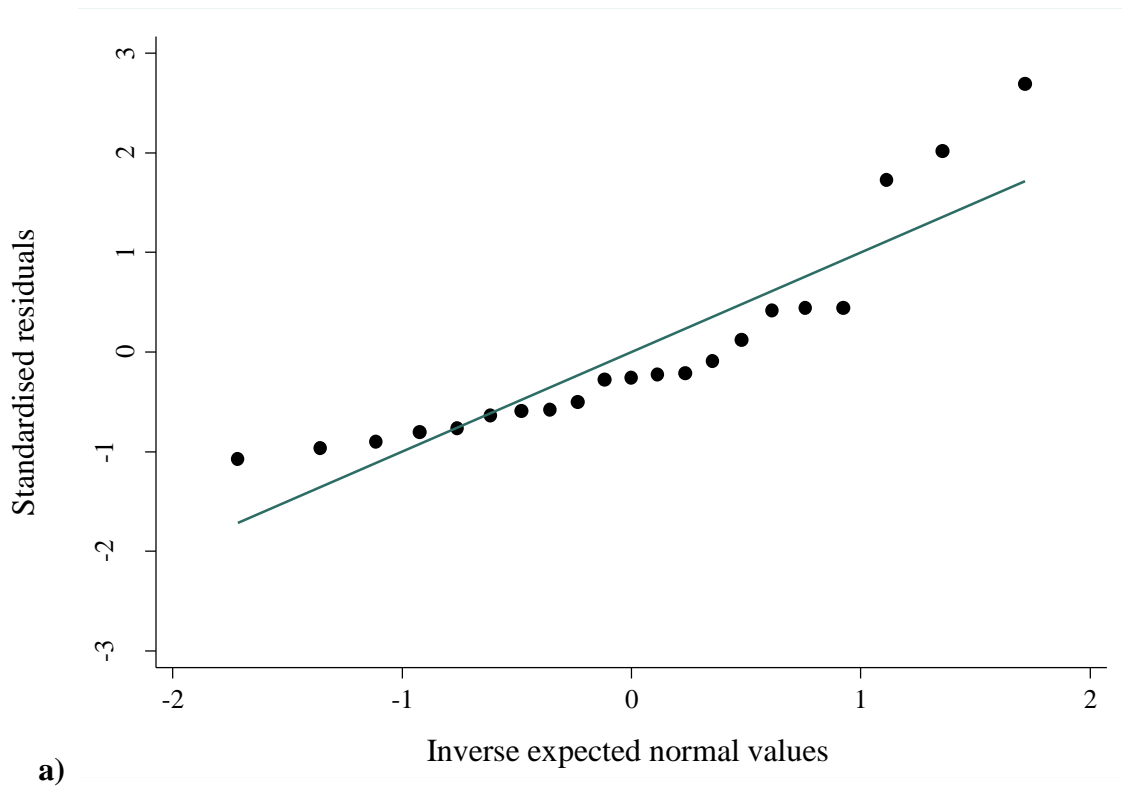


a)

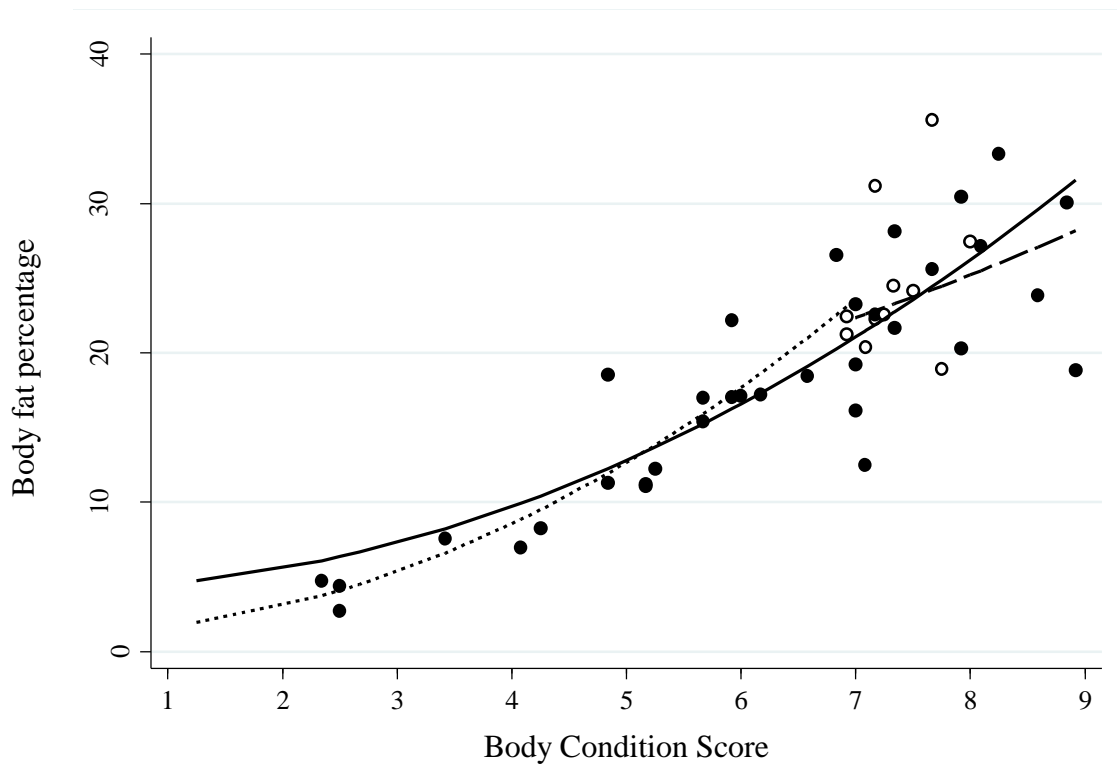


b)

**Figure 6.4:** Scatterplots of the standardised residuals against predicted values for the linear regression models based on **a)** ‘non-obese’ ( $K_{ave} < 7/9$ ;  $n = 21$ ) and **b)** ‘obese’ animals ( $K_{ave} \geq 7/9$ ;  $n = 24$ ).



**Figure 6.5:** Quantile-quantile plots of standardised residuals from the linear regression models for **a)** ‘non-obese’ ( $K_{ave} < 7/9$ ;  $n = 21$ ) and **b)** ‘obese’ animals ( $K_{ave} \geq 7/9$ ;  $n = 24$ ) animals against their respective expected, inverse normal values. Imperfect normality is suggested by deviation of data points from the line.



**Figure 6.6:** Scatterplot of body fat percentage (determined from deuterium oxide dilution) and body condition score ( $K_{ave}$ ). Black circles denote Welsh Mountain ponies ( $n=34$ ); white circles denote other breeds ( $n=11$ ). The solid black line illustrates the model for prediction of body fat from  $K_{ave}^2$  for all animals ( $n = 45$ ). The dotted line shows the model for prediction of body fat percentage from  $K_{ave}^2$  when only ‘non-obese’ animals ( $K_{ave} < 7/9$ ;  $n = 21$ ) were included. The dashed line shows the model for prediction of body fat percentage from  $K_{ave}^2$  when only ‘obese’ animals ( $K_{ave} \geq 7/9$ ;  $n = 24$ ) were included. Note the greater scatter of points at the higher BCS values.

## 6.5 Discussion

In agreement with earlier work, the results of this larger study (45 horses and ponies), demonstrated a non-linear association between equine BCS and body fat content (Dugdale *et al.*, In Press a). However, in contrast to our previous results (7 pony mares) and those of Martin-Rosset and colleagues (20 French sport horses, 2008), the relationship was most appropriately described by a power function (body fat %  $\propto K_{ave}^2$ ) as opposed to an exponential function (body fat %  $\propto e^{0.46K_{ave}}$ ) of BCS. This difference was likely due to the larger sample size, although the heterogeneity of breed, age and sex of the study animals may also have affected the results. Further work on different subgroups of animals would enable better detection of any breed, sex, age or seasonal differences in the relationship between BCS and body fat content.

The initial validation of the D<sub>2</sub>O dilution technique for determination of body fat in *Equidae* was limited to the study of mature Welsh mountain pony mares of  $K_{ave}$  1-7/9. Although the technique has been widely used in several species including man, without recourse to any comparable test of its suitability, it remains worthy of consideration that this study used a broader variety of breed, sex, age and BCS of horses and ponies than the initial validation (Dugdale *et al.*, In press b).

The two negative values of body fat, calculated for two thin animals, were excluded from the present data analysis because further evaluation of the lean tissue hydration factor applicable to such individuals is warranted (Dugdale *et al.*, In press b). That such lean animals have relatively greater tissue water content and a greater gut water pool may be responsible for over-estimation of lean tissue mass when the ‘universal’ lean tissue hydration constant is applied (0.732, Pace and Rathbun, 1945).

Subsequent under-estimation of body fat occurs consequent upon its calculation by subtraction of lean tissue mass from actual body mass. Although equations were previously suggested for correction of body fat under-estimation, their application did not resolve the negative fat value for one of the present two ponies (Dugdale *et al.*, In press b). It was therefore decided not to apply that equation to any of the body fat values in the present study, but to model this larger data set on the raw values.

The application of a BCS system developed for Quarter horse broodmares to other breeds, and indeed a mixture of breeds simultaneously, might be cautioned (Suagee *et al.*, 2008; Dugdale *et al.*, In Press a & b; present study). In support of this commonly used BCS technique, earlier studies in this series (Dugdale *et al.*, In Press a) demonstrated a near perfect linear association between BCS ( $K_{ave}$ ) and total body soft tissue (flesh), suggesting that Kohnke's modified version of Henneke and colleagues' score may at least be applicable to pony mares for assessment of total 'flesh' (Henneke *et al.*, 1983; Kohnke, 1992). When BCS systems were first developed to optimise farm animal welfare and productivity, they were solely intended for assessment of body flesh (Jeffries, 1961). Our recent concern with rising obesity within human and companion animal populations has seen an increasing shift towards the use of BCS systems for the evaluation of body fat content, a purpose for which they were never originally designed (Laflamme, 1997a & b). Interestingly, Burkholder (2000) has noted that if BCS systems can only determine fat percentage to within the nearest  $\pm 10\%$  (with 95% confidence), then this really only allows distinction of three 'fat' classes: thin, moderate and overweight.

For observational, categoric (rather than true measurement) scoring systems such as the BCS system used here, which is presented as a linearly-ordinal scale with 9 discontinuous categories, statistical evaluation after conversion to a continuous scale (by averaging regional scores in accordance with the method's instructions), could be criticised. This is because the nature of the association between the continuous score and what is being predicted, and also the 'weightings' for different body regions, are often unknown (Streiner and Norman, 2008). For statistical correctness, parametric methods should only be applied if the association (between score and predicted variable) is linearly ordinal (e.g. where the difference between scores 1 and 2 is represented by the same percentage change in body flesh or body fat as the difference between scores 8 and 9). For pony mares, total body flesh was indeed linearly associated with  $K_{ave}$  (Dugdale *et al.*, In Press a), so one could argue in favour of the application of parametric statistical methods. Body fat content, however, has been shown to be non-linearly related to BCS (Martin-Rosset *et al.*, 2008; Dugdale *et al.*, In Press a; present study). That the exact nature of the non-linearity, for example whether an exponential or power function best described the association between BCS and body fat content, differed between studies might have been a consequence of the different breeds, BCS systems and actual numbers of animals studied. Further evaluation of present and future BCS systems may enable the development of a truly interval scale for prediction of body fat.

Using Kohnke's system, our earlier work suggested that at higher  $K_{ave}$  there was a reduced ability to discriminate between 'fatness' classifications once animals became overweight ( $BCS > 6/9$ , Dugdale *et al.*, In press a). Indeed, a critical appraisal of the descriptors presented within the BCS system (Table 6.1), highlights the



subjective nature of the scoring system which may be open to individual interpretation. For example, under 'Tailhead', the terms for fat that 'feels soft' (score 6/9), 'is soft' (score 7/9) or is 'very soft' (score 8/9) are difficult to differentiate, even for an experienced observer. In addition, the difference between fat feeling 'rounded off' (score 7/9) or 'bulging' (score 9/9) is not always entirely obvious, and in order that 'fat can be felt' (score 4/9), one could argue that it may already be rounded off and even bulging. Some overlap/redundancy was therefore apparent between descriptors for ranges of scores which would reduce the inter-observer reliability of the scoring system. Indeed, Teasdale and Jennett (1974) reported that for a scale to be generally acceptable as universal, it must be practical to use in a wide range of locations and without training and they emphasised the importance of defining descriptors precisely so that the observer is left in no doubt as to their interpretation. Interestingly, a recent study reported variation in scoring between 'veterinarians', suggesting that even more variation might be apparent between scorers when horse owners perform the scoring (Mottet *et al.*, 2008).

The present study enabled investigation of the sensitivity of BCS to predict fat content over a wide range of BCS values. A cut-off value of 7/9 was determined after observation of flaring (a wider residual variation) in  $K_{ave}$  at these higher scores. Subsequent modelling of 'non-obese' and 'obese' animals supported a greatly reduced association between body fat content and  $K_{ave}$  at scores of 7 or greater, emphasising that once animals are 'obese', subjective scoring appears to be an insensitive tool to determine their actual body fat content. Earlier work on a group of overweight and obese ponies, for which dietary intake was restricted for 3 months (1% of actual body mass as daily dry matter intake), showed clinically insignificant reductions in BCS

( $K_{ave}$ ) despite significant body mass loss (Dugdale *et al.*, 2010). Not only would these ponies have been on the steep region of the curve, wherein a large change in body fat content would be represented by a relatively small change in BCS, but also they were on the least sensitive part of the curve to determine accurate body fat content, thus rendering BCS a poor tool for monitoring early weight/fat loss (Dugdale *et al.*, 2010). Interestingly, a similar cut-off value of BCS 7/9 has been reported for prediction of pasture-associated laminitis, such that animals with BCS 7 or greater are at increased risk of developing laminitis (Carter *et al.*, 2009). Such an ability to be able to differentiate those animals at risk of suffering this painful and debilitating condition should trigger the application of preventative measures and represents an important step towards improving equine welfare. However, consistency in inter-observer application of BCS systems must be guaranteed before the practical usefulness of such findings can be fully realised.

Measurement error may be random or systematic. Whereas systematic measurement error generally does not average out to zero, random measurement error can do and is often ignored as a source of data misinterpretation (Hutcheon *et al.*, 2010). Random error in determination of the *outcome* variable has minimal effect on the regression coefficient itself but widens the standard error of the estimate. A larger sample size and/or increased number of measurements taken from each subject would reduce the effect of random error on the model. In contrast, random measurement error in determination of the *predictor* variable ( $K_{ave}$  in this instance), could result in an underestimation of effect called regression dilution bias (Hutcheon *et al.*, 2010). However, determination of the sources and magnitude of such errors, in order to minimise them, is not easy for such a subjective appraisal as body condition scoring.

Perhaps a good place to start would be the development of unambiguous descriptors? Indeed, with this in mind, a critical review of the descriptors of the BCS system (Kohnke, 1992; after Henneke *et al.*, 1983) was undertaken with the help of lay horse owners which aimed to reduce the ambiguity of, and redundancy amongst, descriptors. The outcomes of this survey, in combination with personal experience, were used to develop the framework for a more concise and unambiguous scoring system (Annex 1). Furthermore, to avoid the problems of uneven weighting between scoring sites, an additive score was composed, rather than one where scores are inappropriately averaged between sites. Arbitrary weightings have, however, been applied to the descriptor categories for each site in an attempt to linearise the final score which would make statistical, and indeed clinical, interpretation easier. The validity of these values and indeed of the whole scoring system requires testing and forms part of our ongoing studies.

## **6.6 Conclusion**

This study highlighted the subjective nature of BCS and its poor ability to predict fatness once an animal is already fat. BCS descriptors may need refining in order to improve BCS systems to sub-categorise fat animals, although once fat (BCS  $\geq 7$ ), these animals are likely to be at increased risk of obesity-related disease.

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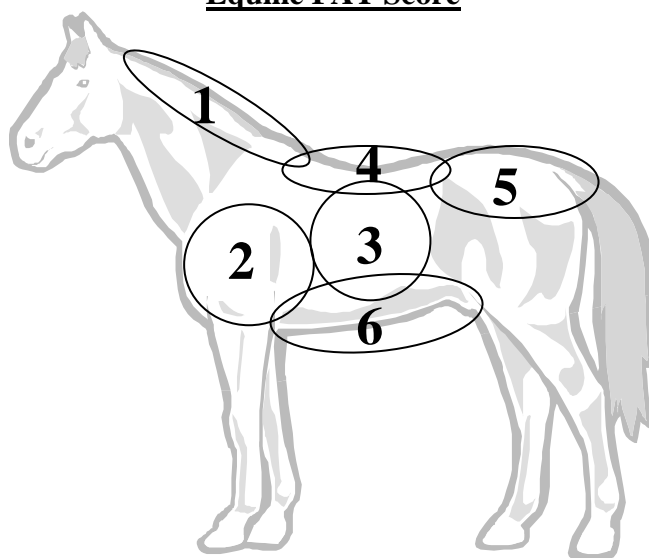
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# **Annex 1**

## **Novel Equine FAT Scoring system (E-FATS)**

### Equine FAT Score

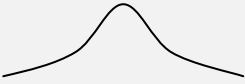

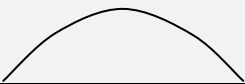
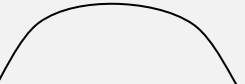
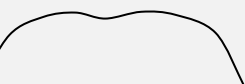



1. Neck top-line (select one category)			Score
Ewe neck (concave top-line).			0
Straight.			1
Slight convex curve (slight crest).			5
Very convex (huge crest); may flop to one side. May have wrinkles along top-line. Fat pads may be present on the 'sides' of the neck.			15
Score for Neck (a) (Stallions scoring 15; subtract 5) =			

2. Shoulder (select one category)	
Shoulder bones prominent, very angular and easily felt.	0
Shoulder bones less angular but still felt with gentle pressure. No fat pads around shoulder.	2
Slight fat pads behind and possibly in front of shoulder.	5
Large fat pads present, forming obvious bulges, behind and in front of shoulder.	10
Massive bulging fat pads surround the shoulder.	20
Score for Shoulder (b) =	

3. Ribs (select one category)	
Ribs easily felt when stroked lightly.	0
Ribs still felt with gentle pressure.	2
Ribs require moderate-to-firm pressure to feel.	5
Ribs very difficult to feel even with firm pressure.	10
Fat pads behind shoulder may merge with those over ribs to form blanket of fat.	20
Score for Ribs (c) =	

4. Back (select one category)	
Sharp ridge along spine.	0
Slight ridge along spine.	1
Back more or less level.	3
Deep midline gutter along back.	5
Score for Back (d) =	

5. Rump (select one category)		
Pelvic bones project sharply; skin tight over bones.	Rump falls away either side of spine. 	0
Pelvic bones easily felt; but some 'fleshy' cover.	Rump angular but less sharp. 	1
Pelvic bones rounded and indistinct.	Rump slightly rounded. 	3
Pelvic bones hard to feel. Fat pads around tail base.	Rump very rounded. 	10
Pelvic bones hard to feel. Fat pads around tail base.	Rump bulges slightly either side. 	20
Pelvic bones and tail base buried in large amounts of fat.	Rump bulges either side. 	25
Score for Rump (e) =		

6. Miscellaneous	
Fat pad/s on underside of belly.	5
Wobbly fat pad over point of elbow.	5
Fatty udder/sheath.	5
Score for Miscellaneous (f) =	

Neck (a)	Shoulder (b)	Ribs (c)	Back (d)	Rump (e)	Miscellaneous (f)	TOTAL (a + b + c + d + e + f)
If Cob-type, subtract 10 from Total						
FINAL GRAND TOTAL =						



DATE.....

Scorer's Identification.....

Animal's name.....

Animal's age.....

Animal's breed.....

Animal's sex.....

**Preliminary interpretation (scale from 0 to 100)**

**0 to 7 = Too thin**

**8 to 25 = Moderate**

**26-40 = Overweight**

**41-75 = Obese**

**>75 = Morbidly Obese**

# **Chapter 7**

## **Concluding Discussion and Future Directions**

While the work contained in this thesis has greatly improved our understanding of the biology of white adipose tissue in the Native pony, many questions have also been raised which remain to be addressed. The pony has, however, been revealed as a highly seasonal animal that demands specific attention if it is to be maintained within ‘modern’ lowland husbandry systems.

## **7.1 Seasonality**

It was no surprise to determine seasonal differences in appetite and maintenance energy requirements in non-obese Native ponies (Dugdale *et al.*, In Press a), since earlier work in the same breed had demonstrated such photoperiodically-entrained changes (Fuller *et al.*, 2001). However, it was intriguing that despite clear evidence of continued photoperiodic entrainment with respect to pelage growth, we could determine no expression of seasonal variation in terms of appetite and ‘growth’ (fat deposition) in obese ponies. It is tempting to speculate that the effect of photoperiod on the hypothalamo-pituitary axis’ ability to mediate seasonal metabolic changes may become somewhat dulled in the obese state because of altered interactions between, for example, leptin and the hypothalamo-pituitary-adrenal and thyroid axes (Van Cauter *et al.*, 1997; Ahima *et al.*, 2000; Flier *et al.*, 2000; English *et al.*, 2002; Murphy, 2010). No doubt that insulin resistance and leptin resistance are also important.

## **7.2 Body composition and basic adipobiology: WAT content and distribution**

In common with other species, the white adipose tissue (WAT) content of pony mares from a wide range of body conditions, was the most variable body component, comprising up to 1/3<sup>rd</sup> of live body mass (Lohman, 1971; Webb and

Weaver, 1979; Dugdale *et al.*, In Press b). The WAT was partitioned almost equally between internal (body cavity) and external sites (subcutaneous, intermuscular, intramuscular) (Dugdale *et al.*, In Press b). When *ad libitum*-fed, ponies of non-obese outset body condition gained considerable body mass, a substantial part of which was fat, especially when weight was gained during summer months (Dugdale *et al.*, In Press a). Moreover, there was a tendency for fat to be deposited preferentially at internal sites during the summer, whereas subcutaneous sites were apparently favoured during winter, perhaps for a thermoregulatory advantage (Dugdale *et al.*, In Press a).

The distribution of fat between different body depots ('patterning') and the order of its deposition and withdrawal from these depots, can be extremely variable, even between relatively homogeneous individuals (Pond, 1998; Reed *et al.*, 2006). However, for farmed ruminants of the same breed, kept under similar husbandry conditions, and wild deer in the same geographic location, the order of fat deposition or withdrawal from different depots was relatively constant, although was not necessarily the exact opposite when body condition was increasing compared with when body condition was decreasing (Riney, 1955; Russel *et al.*, 1968 & 1971; Butler-Hogg, 1984; Butler-Hogg *et al.*, 1985; Gregory *et al.*, 1998). For these ruminants, it appeared that the subcutaneous and omental fat deposits were the last to accrete during growth and fattening and were among the first to deplete during fat mobilisation, with bone fat depleting much later. Whether subcutaneous fat depths altered before, alongside or after omental fat changes during fat accretion and depletion, however, appeared somewhat variable. From our studies in ponies, the subcutaneous and retroperitoneal depots certainly had huge capacity for fat storage

(Dugdale *et al.*, In Press b). For thinner ponies, omental fat was much reduced and only in the thinnest pony did bone fat decrease (Dugdale *et al.*, In Press b). Further studies would be required for each age, breed and sex of horse and pony, to determine the exact order of fat deposition at the different depots during fattening, and of its mobilisation during weight loss under defined conditions. Although advanced imaging techniques such as dual energy X-ray absorptiometry, computed tomography and magnetic resonance imaging are not applicable to horses and ponies, they can be used in smaller species including humans to study fat patterning (Goodpaster *et al.*, 1999). Indeed, with these techniques, the subcutaneous adipose tissue of humans can itself be observed to form two layers, separated by fascia, which are differentially active (Enevoldsen *et al.*, 2001; Ross and Janssen, 2005).

In horses and ponies, only the superficially-accessible fat depots are readily amenable to study over real-time by techniques such as ultrasound imaging and biopsy. The main problem with ultrasonic measurement of superficial fat depths is the lack of consensus as to the exact anatomical locations for measurement, making comparisons between studies impossible (Westevelt *et al.*, 1976; Kane, 1987; Gentry *et al.*, 2004).

As for biopsies of WAT, adipocytes from different depots have varying activities and physiological roles (Pond, 1998; Burns *et al.*, 2009; Suagee *et al.*, 2010) but further elucidation of these is required before any depot-specific roles during fat deposition and mobilisation can be understood. Within WAT, however, it is not just the adipocytes that produce adipokines (the most studied being leptin and adiponectin which are reciprocally affected by changes in WAT mass); the stromovascular

component may be responsible for as much as 90% of the total adipokine production (Trayhurn and Beattie, 2001; Fain *et al.*, 2004). While the inter-play between the two compartments of WAT (the adipocytes and the stromovascular component which includes vascular endothelium and macrophages) may well be depot-specific, there may also be inter-depot signalling. The complexities of WAT activity and regulation on a depot-specific and whole-body scale remain to be determined in different species.

### **7.3 Measures of adiposity *in vivo***

#### **7.3.1 Body condition scoring**

The exploration of a horse-specific body condition scoring system in Native pony mares strongly suggested a non-linear relationship between body condition score (BCS) and body fat content (Dugdale *et al.*, In Press b and c). Such non-linearity does not enable easy translation of this subjective appraisal into a reliable tool for the estimation/prediction of body fat content because prediction usually relies on linear regression analysis (Guo *et al.*, 1996). In addition, animals of high BCS differed more widely in their fat content than animals of lower BCS values which complicates translation of BCS values into body fat content even if the non-linear relationship is accounted for. A novel pony BCS system has since been developed (Annex 1), but how it relates to body fat content and whether it can be applied to other pony sexes and breeds, remain to be determined and are the subject of on-going studies.

By its very nature, body condition scoring only allows appraisal of superficial ‘flesh’ (soft tissues, including fat), yet ponies carried approximately half of their total body fat internally (Dugdale *et al.*, In Press b). Whether horses and ponies can also

distribute their fat less evenly between internal and external sites remains to be determined, but people certainly can, whilst appearing non-obese externally. More worryingly, these ‘normal weight, metabolically obese’ people (or ‘TOFI’: thin on the outside, fat on the inside), can suffer the same medical consequences of obesity as more readily-recognised, externally obese people (St-Onge *et al.*, 2004; De Lorenzo *et al.*, 2007). Body condition scoring should therefore perhaps be complemented by some method of appraisal of internal fat. To date, only abdominal retroperitoneal fat depths have proved amenable to routine appraisal for this purpose. Reliance on a single internal deposit, whilst useful, fails to account for between-animal differences in fat patterning and further methods to quantify other reserves should be explored to complement this measure.

Burkholder (2000), has previously cautioned the clinical usefulness of BCS systems, suggesting that, at best, they can only ascribe an animal to one of three classes: thin, average or overweight, because within most BCS systems, each BCS value can only predict body fat content with a 95% confidence interval of  $\pm 6$  to 10%. In particular, he emphasises that it is the mean body fat content of all those animals assigned a particular score that becomes ‘the’ body fat content associated with that score, which already introduces an error due to potentially large inter-individual variation. How a BCS system relates to body fat content, therefore also becomes a function of the particular study population; the larger that population, the better.

Although body condition scoring will always remain a subjective tool, its application ‘in the field’, even if only to enable an initial coarse assignment into ‘thin, average or overweight’ categories, should also be sufficient to encourage long-term

monitoring in order to improve equine health and welfare. Until a more objective, practical and cheap alternative method is validated, BCS systems will likely remain a commonly-used tool for the assessment and monitoring of body fatness in horses and ponies. To this end, ensuring that the BCS system in use is the most accurate and precise available has been a key outcome of this work. A novel BCS system was therefore composed (Annex 1) which has several novel features. The descriptors were selected/modified after evaluation by lay horse-owners in order to reduce confusion and ambiguity in the terms employed. The final descriptor choice and overall ease of application of the E-FATS system were again evaluated by the same, and other, lay horse-owners to ensure the ‘user-friendliness’ of the system. The scoring system was designed to be additive, so that the overall score is the sum of the individual scores and not an average, thus avoiding the problem of averaging a potentially non-linear interval score. However, empirical weightings were applied, assuming a non-linear association with body fat content, in an attempt to create a linear relationship between the total score and body fat content. These weightings will no doubt need some corrections once sufficient data have been gathered from a large population of animals of different body conditions. While the E-FATS system was initially intended to be pony-specific, it will be tested on horses as well as ponies and across different breeds and sexes, deuterium oxide dilution being used for the measurement of actual body fat.

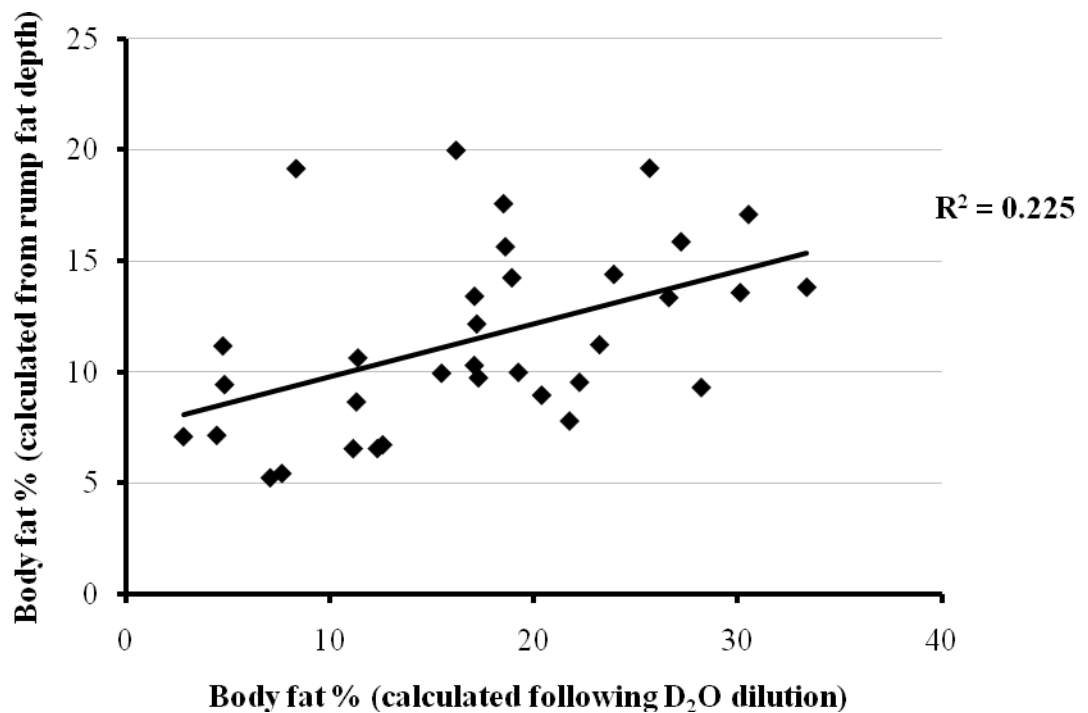
### **7.3.2 Morphometric and ultrasonographic measures of body fat**

Several studies have concluded that objective, morphometric measures such as heart girth, umbilical girth and neck circumference, especially when indexed to withers height, were positively related to body fat content (Carter *et al.*, 2009a;



Dugdale *et al.*, 2010, In Press a and b). Neck circumference, however, was found to be less useful during weight loss (Dugdale *et al.*, 2010). While morphometric measurements may complement body condition scoring, their use as sole predictors of body fat content should be cautioned against because of the lack of reference values for all the different breeds, ages and sexes of horses and ponies. However, until such reference values/ranges can be determined, morphometric measures, particularly those of heart and belly girths, still provide useful information for the practical monitoring of changes in body condition.

Ultrasonographic measurements of the depth of various superficially-accessible fat depots offer another possible objective technique to predict total body fatness (Westervelt *et al.*, 1976). Not all studies, however, have agreed with the results of Westervelt and colleagues (Kane, 1987; Gentry *et al.*, 2004; Dugdale *et al.*, 2010), suggesting that their original predictive equations should be used with care (Guo *et al.*, 1996). Indeed, ultrasonic data from our various pony studies poorly predicted total body fat when using Westervelt's equation for ponies (Figure 7.1), which led me to agree with Kane (1987), that the anatomical site chosen for measurement must be precisely defined (Dugdale *et al.*, 2010, In Press a and b). That many studies have reported, and continue to report equine body fat percentage calculated on the basis of the equations described by Westervelt and colleagues (1976) and Kane (1987), suggests that many authors are unaware of the potential errors associated with this technique. This author hopes to publish a critique on this topic in order to discourage the continuation of this malpractice.



**Figure 7.1:** Body fat percentage calculated from rump fat depth using the formula from Westervelt *et al.* (1976; Body fat percentage =  $3.83 + 5.58 \times \text{rump fat[cm]}$ ), was regressed on body fat percentage determined from deuterium oxide dilution in Welsh pony mares. Data were gathered contemporaneously ( $n = 34$ ). The linear regression line and coefficient of determination is presented ( $R^2$ ).

Similarly to morphometric measurements, ultrasonic fat depth measurements may provide a useful complement to body condition scoring but, again, they are not consistently related to body fat content across all breeds, ages and sexes. Therefore they are likely to be most useful for monitoring changes over time. However, the time-frame and circumstances of monitoring any changes may be important to consider because a recent study of dietary restriction (1.25% of body mass as daily dry matter intake), and weight loss, in a mixed group of horses and ponies,

demonstrated an increase in both rump fat and rib fat thickness over the whole 16 week study (Curtis *et al.*, 2010). One obese pony in a previous study demonstrated a similar increase in rump fat thickness (Dugdale *et al.*, 2010). Hypotheses for such increases in subcutaneous fat depths include firstly, angiogenesis / increased vascularity within the white adipose tissues to facilitate lipolysis and secondly, possible fat redistribution between depots during this winter season when subcutaneous depots are preferentially favoured (Dugdale *et al.*, In Press a), especially if some dietary essential or conditionally-essential nutrient, such as L-carnitine, was relatively lacking, thus reducing the potential for mitochondrial beta oxidation of fatty acids (in tissues such as liver or muscle) which would require their re-storage in WAT. However, although dietary protein (lysine and methionine are required for carnitine synthesis), was possibly marginally insufficient in one study (Dugdale *et al.*, 2010), supplementation was provided in the second (Curtis *et al.*, 2010). Regardless of the measured increase in subcutaneous WAT in some locations in these animals, a reduction in total body fat content was measured by the deuterium oxide dilution technique (Curtis *et al.*, 2010; Dugdale *et al.*, 2010). As previously stated (section 7.2), further studies are therefore required to elucidate the processes determining regional fat deposition and mobilisation in horses and ponies.

### **7.3.3 Bioelectrical impedance analysis**

Another practical and objective technique for the assessment of body fat is bioelectrical impedance analysis (BIA). In multi-frequency BIA (MF-BIA), electrical currents of several different frequencies are used in order to optimise the differentiation of lean and fatty tissues because high frequency currents can penetrate cell membranes, whereas low frequency currents cannot (Kyle *et al.*, 2004). Stored

triglycerides, which are anhydrous, remain impermeable to electrical currents. Total body water and extracellular water can therefore be estimated and calculation of total body fat depends upon use of the universal lean tissue hydration factor (0.732) first reported by Pace and Rathbun (1945). Although there has been much debate about this ‘universal’ lean tissue hydration constant, most authors tend to agree with the original value (Wang *et al.*, 1999). Nevertheless, individuals at either end of the body condition spectrum may be exceptions and Sheng and Huggins (1979) have cautioned that even the small range of lean tissue hydration values found across many species (70 to 76%), can incur large errors in calculation of body fat from total body water. These same criticisms also apply to calculations of body fat derived from measurements of total body water using isotope dilution techniques, especially in herbivores where gut water may represent a relatively large proportion of the total body water (Dugdale *et al.*, In Press c). However, despite these potential limitations, D<sub>2</sub>O dilution-derived body fat content has proved to be a useful proxy measure of total body fat content; and although, to date, we have not been able to demonstrate similar agreement for BIA-generated measures, the apparent simplicity of this methodology warrants further study.

In practice, BIA techniques require that electrical currents are passed through the body between surface contact or hypodermic needle electrodes (in *Equidae*, either “fore-limb to hind-limb” or “poll to dock”) enabling the electrical impedance (the vector between the electrical resistance and reactance) to be determined (Forro *et al.*, 2000; Fielding *et al.*, 2004, Van der Aa Kuhle *et al.*, 2008). In the studies of Forro and colleagues (2000) and Fielding and colleagues (2004), the determination of body water compartments by MF-BIA was evaluated in horses. In a further variation of the

technique, bioelectrical impedance spectroscopy (BIS), which depends heavily on mathematical modelling, predicted equine body fat with some success, which, in the opinion of this author was possibly facilitated by the use of hypodermic needle electrodes and a poll-to-dock configuration (Van der Aa Kuhle *et al.*, 2008). In addition, this last group used sodium bromide to measure extracellular fluid space and D<sub>2</sub>O to measure TBW, their study being the best attempt so far to validate bioelectrical impedance as a technique to measure water compartments and fat in *Equidae*. Despite these early indications of a promising portable method to determine body fat in horses and ponies, validation of the technique across all breeds and types remains elusive. To this end, however, MF-BIA data (Equistat®) were collected, concurrently with all other subjective and objective measures of fatness, from all the animals used during data collection for this thesis and are due to be analysed forthwith.

#### **7.3.4 Deuterium oxide dilution**

Rigorous carcase dissection and chemical composition analysis enabled a comparison of deuterium oxide (D<sub>2</sub>O) dilution, as a non-invasive, indirect method of determining body fat, with these two gold standard, reference techniques in ponies (Dugdale *et al.*, In Press c). Although only a small population was studied, and of one breed and sex, the results suggested that these three methods were all but interchangeable for the determination of body fat content, despite concerns over use of the ‘universal’ lean tissue hydration factor in these herbivorous animals (with a relatively large reservoir of gut water), of differing body fat content and gut water pool size (Dugdale *et al.*, In Press c; section 7.3.3 above). Although prior to its validation, several authors had reported the use of the D<sub>2</sub>O dilution technique to

estimate equine body fat content (Van der Aa Kuhle *et al.*, 2008; Carter *et al.*, 2010; Dugdale *et al.*, 2010), it should now become a more widely accepted technique, being the only current, valid, non-invasive method of body fat prediction in *Equidae*.

#### **7.4 Pathophysiology of obesity**

The major biochemical and physiological consequences of obesity are inflammation and insulin resistance, with adipokines and free fatty acids being some of their main mediators (Bastard *et al.*, 2006; Tilg and Moschen, 2006; Vick *et al.*, 2007). However, not all insulin resistant individuals are obese as various stressors (starvation, injury, sepsis and pregnancy) can also evoke it (Breukink, 1974; Van den Berghe, 2004; Kronfeld *et al.*, 2005a; Geor, 2010). Neither are all obese individuals insulin resistant, although regional obesity (including TOFI individuals) is usually a pre-requisite (Leslie, 2005; Karelis, 2008). For humans, the clinical consequences of insulin resistance have been recognised for some time; indeed as early as 1981, Hanefield and Leonhardt first used the term ‘metabolic syndrome’ to describe “the joint incidence of obesity, hyperlipoproteinaemia, diabetes, gout and hypertension, together with increased incidence of ischaemic cardiovascular disease, fatty liver and cholelithiasis”. Since then, however, several synonyms and definitions/inclusion criteria (from ‘android obesity’ to ‘dysmetabolic syndrome X’ to ‘the deadly quartet’) have been proposed which, according to some authors, have only served to confuse matters (Reaven, 2004; Kahn *et al.*, 2005; Leslie, 2005; Reisin and Alpert, 2005; Reaven, 2006). In light of this confusion, reversion to the simpler term ‘insulin resistance syndrome’ has been suggested in recognition of the fact that it is the insulin resistance itself which underlies the pre/sub-clinical and eventually clinical features of disease, the clustering of which is then characterised by terms such as the cardio-

metabolic syndrome (Gill *et al.*, 2005; Hutley and Prins, 2005; Reaven, 2006). The term insulin resistance therefore appreciates the underlying pathophysiology, whereas some form of the term ‘cardio-metabolic syndrome’ facilitates clinical diagnosis for the purposes of improving public health (Reaven, 2005).

It is with much interest, therefore, that the term ‘equine metabolic syndrome (EMS)’ has recently been adopted by the American College of Veterinary Internal Medicine (ACVIM Consensus Statement: Equine Metabolic Syndrome, Frank *et al.*, 2010), perhaps prematurely (Kronfeld, 2003), to describe a collection of risk factors for the development of laminitis: insulin resistance, obesity/regional obesity and previous clinical/subclinical laminitic episodes which have occurred in the absence of recognised causes such as grain overload, metritis or colitis. As with the situation in humans, however, additional phenotypic criteria have also been described. For horses and ponies, these include: hypertriglyceridaemia or dyslipidaemia, hyperleptinaemia and arterial hypertension.

Whereas dysregulation of lipid metabolism, recognised as overt dyslipidaemias, is a common feature of the human metabolic syndrome, it is less consistently demonstrated in *Equidae* (Grundy, 1999; Lago *et al.*, 2009; Dugdale *et al.*, In Press a). Relative hypertriglyceridaemia has, however, been documented in some obese ponies, including ours (Jeffcott *et al.*, 1986; Freestone *et al.*, 1991; Treiber *et al.*, 2006a; Dugdale *et al.*, In Press a), although serum non-esterified fatty acids were not consistently elevated, possibly due to rapid conversion to triglycerides by the liver (Frank *et al.*, 2006; Treiber *et al.*, 2006a).

A further complication for *Equidae*, is the overlap of the EMS phenotype (regional adiposity, insulin resistance and laminitis), with the clinical signs associated with equine Cushing's disease (PPID: pituitary pars intermedia dysfunction), although differentiation may be possible by considering age of onset (PPID is rare in animals <15yr) and other clinical signs associated with increased ACTH production, hyperadrenocorticism and space-occupying pituitary adenomata within the cranium (polyuria/polydipsia, skeletal muscle atrophy, increased susceptibility to infections, hirsutism, excessive sweating and somnolence) (Menzies-Gow, 2006; Lee *et al.*, 2010).

Overt hyperglycaemia is rare in insulin resistant *Equidae*, although it is possible that varying degrees of relative hyperglycaemia occurs in some of the more severely affected animals (personal observations). Glucose uptake by the tissues is regulated by both insulin-dependent and non-insulin-dependent mechanisms. Facilitated diffusion aids glucose uptake in most tissues, although sodium/glucose co-transport (secondary active transport) is also important for glucose absorption in the gut and renal tubules. The glucose transporters required for facilitated diffusion belong to the GLUT family of proteins; there are at least 12 (Gorovits and Charron, 2003; Zhao and Keating, 2007). Table 7.1, below, summarises the known functions of the main GLUTs to date (Klip *et al.*, 1994; Gorovits and Charron, 2003; Zhao and Keating, 2007; Cheeseman, 2008; Simpson *et al.*, 2008). Other glucose transporters also exist, serving only to indicate the complexity of glucose homeostasis (Wood and Trayhurn, 2003). GLUT4 expression (mRNA and protein) in different WAT depots and muscles was to be reported herein but sadly was not possible. It is hoped that this will be revisited in future using animals of different BCS and insulin sensitivities.



Glucose itself mediates the cellular uptake of glucose via GLUT1 and GLUT3.

Insulin mediates muscle and adipose tissue uptake of glucose via GLUT4, by stimulating the translocation of vesicles containing GLUT4 to the cell membrane and increasing their rate of exocytosis (and therefore recruitment of GLUT4 into the membrane), whilst also decreasing their rate of internalisation (Shepherd and Kahn, 1999; Ganong, 2003; Klip *et al.*, 1994; Karnieli and Armoni, 2008). Impaired regulation of GLUT4 gene expression and function occurs with insulin resistant states (Karnieli and Armoni, 2008).

**Table 7.1:** The location and function of the main mammalian facilitative glucose transporters which belong to the GLUT family of proteins that facilitate glucose uptake by diffusion.

Glucose transporter	Tissue location	Insulin-responsive	Function
GLUT1	Ubiquitous. Importantly RBC & cerebral blood vessels  Muscle GLUT1 shows no response to insulin whereas WAT GLUT1 may increase very slightly with exposure to insulin.	No	Basal glucose uptake. Transporters are always present in cell membranes. Hyperglycaemia normally results in down-regulation of GLUT1 expression; hypoglycaemia resulting in up-regulation. Cytokines, hypoxia, angiotensin II, endothelin I and various growth factors can upregulate expression and membrane localisation of GLUT1 in e.g. neurones, vascular endothelium and vascular smooth muscle cells, which may lead to glucotoxicity.
GLUT2	Liver, renal tubular cells, enterocyte basolateral membrane, pancreatic $\beta$ cells	No (but expression of receptors may be insulin-dependent; high insulin may reduce hepatocyte membrane GLUT2 expression)	Pancreatic $\beta$ cell glucose sensor
GLUT3	Predominantly neurones	No	Basal glucose uptake Cytokines, hypoxia, angiotensin II, endothelin I and various growth factors can upregulate expression and membrane localisation of GLUT3 in neurones, which may lead to glucotoxicity.
GLUT4	Skeletal muscle, cardiac muscle, brown adipose tissue, white adipose tissue	Yes In muscle cells, hypoxia and muscular contraction can also stimulate translocation (of a separate pool of GLUT4 vesicles) to the sarcolemma	Insulin-stimulated glucose uptake. Under basal conditions, transporters are sequestered in intracellular vesicles. Insulin stimulates their translocation to the cell membrane.  Thyroid hormones may also increase GLUT4 expression in muscle and WAT.
GLUT5	Human enterocyte luminal and basolateral membranes, testis, kidney, brain, skeletal	No	Fructose transporter

	muscle?, adipocytes?		
GLUT6	Many cells incl. spleen, leukocytes, brain	No	A pseudogene, no protein product
GLUT7	Liver, intestinal tract, testis		Intracellular glucose transporter:- Glucose-6-phosphate transporter in endoplasmic reticulum. Hexose transporter in GI tract.
GLUT8	Testis, blastocytes, mammary gland, low expression in many other tissues incl muscle and fat	Only in blastocyst	Maturation of spermatozoa, insulin-responsive glucose transport in blastocyst
GLUT9	Liver, kidney, placenta	No	Glucose transport
GLUT10	Mainly liver and pancreas, also heart, lung, kidney, skeletal muscle	No	Yet to be proven
GLUT11	Mainly skeletal muscle and myocardium	No	Fructose transporter
GLUT12	Mammary gland, heart, skeletal muscle, fat	Yes?	Yet to be proven

Blood glucose controls insulin secretion in a concentration-dependent manner (Henquin, 2000). Insulin's many effects (energy homeostasis, electrolyte balance, tissue growth and maturation, vasoregulation and immuno-modulation), are mediated by the insulin receptor, which belongs to a subfamily of tyrosine kinases (Lee and Pilch, 1994; Saltiel and Kahn, 2001).

The intracellular insulin signalling pathways diverge: one pathway results in metabolic effects (e.g. glycogen storage), through activation of phosphatidylinositol 3 kinase (PI(3)K); whereas the other pathway results in mitogenic effects (e.g. vascular endothelial repair and health), through activation of various isoforms of mitogen activated protein kinase (MAPK) (Kim *et al.*, 2006). The insulin resistance (of skeletal muscles, adipose tissues and liver [the main up-takers of glucose]), which accompanies obesity tends to occur both at the level of the insulin receptor (e.g. receptor down-regulation or desensitisation), and also 'downstream' of the receptor, particularly at the level of the intracellular metabolic pathways (Matthaei *et al.*, 2000;

Gill *et al.*, 2005; de Luca and Olefsky, 2008). The mitogenic pathways may therefore become relatively over-active which may be partly responsible for some of the adverse vascular effects of insulin resistance (Jiang *et al.*, 1999; Cusi *et al.*, 2000; Kim *et al.*, 2006).

The terms ‘insulin resistance’, its reciprocal ‘insulin sensitivity’, and ‘glucose tolerance’ tend to be used rather loosely, especially in the equine literature (Hoffman *et al.* 2003; Kronfeld *et al.*, 2005b). Insulin sensitivity can be influenced by several factors including insulin itself (Kolterman *et al.*, 1979; Rizza *et al.*, 1985), other hormones and cytokines including adipokines (Bastard *et al.*, 2006), food components (Storlien *et al.*, 2000; Kopp, 2003; Schmidt and Hickey, 2009), food withholding (Breukink, 1974), and exercise (Mikines *et al.*, 1988; Freestone *et al.*, 1992; Marin *et al.*, 1993; Pratt *et al.*, 2007; Treiber *et al.*, 2006b; Schmidt and Hickey, 2009; Carter *et al.*, 2010). The pro-inflammatory state associated with obesity (increased WAT mass and the adipokines produced), appears to be important in the development of insulin resistance (Bastard *et al.*, 2006; de Luca and Olefsky, 2008), but increased circulating free fatty acids and triglycerides are also involved (Boden and Laakso, 2004; Shenk *et al.*, 2008).

## **7.5 Measurement of insulin resistance**

Insulin resistance is characterised by impaired glucose regulation with a compensatory hyperinsulinaemia (Kahn, 1978). Overt hyperglycaemia (and type 2 diabetes mellitus) is rare in horses, most commonly being the result of the chronic and escalating antagonism of insulin’s effects by cortisol (and possibly other pars

intermedia products) in animals with PPID (Menzies-Gow, 2009; Durham *et al.*, 2009).

Insulin sensitivity (the inverse of insulin resistance [IR]) can be assessed by several techniques. The simplest tests assess non-specific indicators of IR such as basal (fasting) hyperglycaemia and basal hyperinsulinaemia, while the most complex, such as the euglycaemic-hyperinsulinaemic clamp technique, offer truly quantitative assessments of IR (Firshman and Valberg, 2007). In between these two extremes, various proxies have been determined, which, although developed in mainly healthy individuals that complicates their application to other populations, may give some semi-quantitative indication of IR with accuracy between the non-specific and quantitative methods (Treiber *et al.*, 2005). An outline of the tests available is presented in Table 7.2.

**Table 7.2:** Methods of measuring glucose tolerance and insulin sensitivity.

Test	Comments	References
<b>Non-specific methods</b>	Dynamic situation not described in one-off samples. Beware effects of stress.	Firshman and Valberg, 2007; Chumbler <i>et al.</i> , 2009; Muno <i>et al.</i> , 2009; Pratt <i>et al.</i> , 2009; Schmidt and Hickey, 2009
Fasting hyperglycaemia	Can't distinguish inadequate insulin secretion from insulin resistance. Varying degrees of hyperglycaemia exist in insulin resistant animals	Durham <i>et al.</i> , 2009; van der Kolk, 2009
Fructosamine; glycated haemoglobin	Not elevated in moderately hyperglycaemic horses; but not properly evaluated in overtly hyperglycaemic animals. Renal threshold for glucose ~11mol/L Fructosamine decreases with hypoalbuminaemia	Shield <i>et al.</i> , 1994; Jensen, 1995; Misciagna <i>et al.</i> , 2004; Reijerkerk and van der Kolk, 2003; van der Kolk, 2009 Young <i>et al.</i> , 1993 Reusch and Haberer, 2001; Murphy <i>et al.</i> , 1997
Fasting hyperinsulinaemia	Reference ranges not standardised	Treiber <i>et al.</i> , 2005
Glucose tolerance tests -oral (OGTT) -intravenous (IVGTT)	Oral test affected by stress of gavage, rate of administration, gastric emptying, intestinal absorption and incretins. IVGTT avoids these problems and the incretin response,	Mehring and Tyznik, 1970; Jeffcott <i>et al.</i> , 1986; Freestone <i>et al.</i> , 1991 & 1992; Ferrannini and Mari, 2004; Kronfeld <i>et al.</i> , 2005a

	<p>therefore preferred.</p> <p>Glucose intolerance doesn't always co-exist with IR as can occur with insulin deficiency, impaired glucose disposal (glucose- or insulin-mediated) and fat adaptation; therefore also measure insulin or perform insulin stimulation test</p> <p><b>Ponies first shown to be more insulin resistant than horses by such tests, regardless of their BCS</b></p>	
Combined glucose (tolerance) and insulin (sensitivity) test (CGIT)	Not truly quantitative but useful field technique. Blood glucose should return to baseline within 25-45min	Eiler <i>et al.</i> , 2005; Frank <i>et al.</i> , 2006; Funk <i>et al.</i> , 2009
<b>Screening tests and proxies</b>	Reference ranges not established for all breeds/types. Beware of the glucose units – whether mg/dL or mmol/L	Kronfeld <i>et al.</i> , 2005a; Treiber <i>et al.</i> , 2005; Durham <i>et al.</i> , 2008
Fasting Glucose: Insulin ratio	Gives a measure of insulin sensitivity	
Fasting Insulin: Glucose ratio	Gives a measure of insulin secretion	Garcia and Beech, 1986
HOMA-IS	Homeostasis model assessment: insulin sensitivity	
HOMA-β	Homeostasis model assessment: % beta cell function	
QUICKI	Quantitative insulin sensitivity check index	
RISQI	Reciprocal of the insulin square root index	
MIRG	Modified insulin: glucose ratio Was found to be low or paradoxically negative in some highly hyperinsulinaemic animals	Durham <i>et al.</i> , 2008
<b>Quantitative methods</b>	Clamp techniques are not physiologic	Ferrannini and Mari, 1998
Insulin suppression		
Hyperglycaemic clamp		DeFronzo <i>et al.</i> , 1979; Rijnen and van der Kolk, 2003
Euglycaemic, hyperinsulinaemic clamp	Different reference ranges have been determined by different equine studies. One equine study demonstrated better repeatability than MinMod FSIGT. Does not give a measure of beta cell response/insulin secretion	Bergman <i>et al.</i> , 1987; Kaske <i>et al.</i> , 2001; Powell <i>et al.</i> , 2002; Rijnen and van der Kolk, 2003; Kronfeld <i>et al.</i> , 2005a; Pratt <i>et al.</i> , 2005 & 2006; Hess <i>et al.</i> , 2006
Minimal Model of insulin and glucose (and even C-peptide) responses to the insulin-modified frequently sampled IGTT (FSIGT); gives:- Sg, Glucose effectiveness AIRg, Acute insulin response to glucose SI, Insulin sensitivity index DI, Disposition index (=AIRg x SI)	Due to hyperbolic relationship between insulin sensitivity and insulin secretion, their product is constant for a given degree of glucotolerance; this product is the Disposition Index See text	Bergman <i>et al.</i> , 1979; Kahn <i>et al.</i> , 1993; Hoffman <i>et al.</i> , 2003
<b>Other tests of beta cell response (insulin secretion)</b>		
Glucose-dependent arginine response test		Ward <i>et al.</i> , 1984; Larsson and Ahre'n, 1998
Glucagon stimulation test with measurement of insulin's C-peptide		Faber and Binder, 1977; Scheen <i>et al.</i> , 1996; Watanabe <i>et al.</i> , 1989; Fall <i>et al.</i> , 2008

The classically described minimal model (MinMod, Bergman *et al.*, 1979) was later modified by incorporating an injection of exogenous insulin 20min after the injection of glucose. This was to enhance the dynamics of the system and facilitate the modelling. The main disadvantages of the MinMod are that the assumed glucose-mediated glucose disposal ( $S_g$ ) may not be entirely devoid of insulin's influence, and that the exogenous insulin dose required may be much greater in insulin resistant individuals. Its advantages are that it measures both insulin secretion and insulin sensitivity simultaneously and therefore it can differentiate between compensated and decompensated insulin resistance. Hoffman and colleagues (2003) first reported the use of the MinMod in horses, although the glucose and insulin doses were based on human studies (Menzies-Gow, 2009). Recently, however, there has been criticism of the established equine MinMod because of the potential for administered glucose to result in the plasma glucose concentration exceeding the renal threshold and urinary glucose loss is not accounted for in the modelling (Toth *et al.*, 2009). Indeed, compared with the original doses (glucose, 300mg/kg and soluble insulin, 30mU/kg), the optimised test suggests doses of glucose of only 100mg/kg and of soluble insulin of 20mU/kg. Furthermore, when Pratt and colleagues (2005) compared the euglycaemic-hyperinsulinaemic clamp (EHC) technique in horses, considered 'gold standard' in people (Borai *et al.*, 2007), with the 'established' MinMod technique, they found better inter-day repeatability with the EHC.

For two of the studies presented (Dugdale *et al.*, 2010; Dugdale *et al.*, In Press a), the intention had been to quantitatively assess insulin sensitivity using minimal modelling of the insulin-modified FSIGT. Unfortunately, many of the tests, especially of overweight and obese ponies, modelled poorly (data not shown), perhaps because

the insulin doses were not tailored to the individual ponies (some of which were likely highly insulin resistant) (Finegood and Tzur, 1996), and/or because the ponies had continued access to food throughout the tests or for some other, as yet unidentified, reason (Frank, 2009). Only non-specific test results could therefore be presented but it is hoped that re-evaluation of the MinMod data and perhaps measurement of C-peptide, will be made at a future date (Toth *et al.*, 2010).

During consideration of the various methods outlined above, it became apparent that different equine researchers withheld food from the animals for different times, or not at all, before testing, which may complicate comparisons of their results. Breukink (1974) reported that within 24-72h of denied feeding, ponies demonstrated some evidence of increased insulin resistance, with a delayed peak and increased AUC<sub>gluc</sub> after a 24h fast, not thought to be due to changes in gut motility, but possibly partly due to a stress response and/or to changes in gluconeogenic enzymes (Freestone *et al.*, 1991). Excessive starvation periods pre-testing should therefore be avoided but there is no consensus regarding fasting. While there are no data for short (<12h) starvation periods, Treiber and colleagues (2006a) did not withhold food (forage) from their ponies; Breukink (1974) used a 12h fast as 'reference' for a 24h fast; Freestone and colleagues (1992) starved their ponies for 17h prior to testing (by the oral glucose tolerance test); Powell and colleagues (2002) starved their horses for 12h prior to testing (by the euglycaemic-hyperinsulinaemic clamp) and Pratt and colleagues (2007) starved their horses for at least 12h prior to testing. It would seem, therefore, that either allowing continued *ad libitum* access to low glycaemic index forage or pre-test starvation for no longer than 17h, should be suitable strategies, although this hypothesis remains to be tested.

## 7.6 Management of obesity (and insulin resistance)

Whilst it is recognised that WAT mass reflects the long-term balance between energy intake and energy expenditure, there may be some underlying regulation of adipose mass which becomes disturbed when obesity prevails (Prins and O’Rahilly, 1997). Simple, practical, inexpensive, reliable and yet accurate and precise techniques to measure obesity or, indeed, one of its common consequences, insulin resistance, remain to be validated for *Equidae*. However, as individual animals will likely be monitored over time, for the management of obesity (or, at the other end of the spectrum, poor body condition), several techniques may be useful for the owner and practising veterinarian: body condition scoring, body girth measurements, ultrasonic fat depth measurements, and the CGIT (Caltabilota *et al.*, 2009). It certainly appears that horses and ponies of  $BCS \geq 7/9$  require management for weight/fat reduction (Dugdale, thesis chapter 6; Carter *et al.*, 2009b; Geor, 2010).

If measuring obesity still poses problems, what of its management? When Jeffrey Friedman’s group finally identified leptin as the adiposity factor which *ob/ob* mutant mice recognised but could not produce and *db/db* mutant mice produced but could not recognise, it was hoped that leptin could be a panacea for the treatment of obesity (Zhang *et al.*, 1994; Coleman, 2010). Most obese people, however, become leptin resistant, for reasons as yet not understood (Considine *et al.*, 1996; Schwartz *et al.*, 2000; Coleman, 2010). Blood leptin concentrations therefore correlate with body fat percentage in people (leptin being produced by adipocytes in proportion to total WAT mass), (Considine *et al.*, 1996). Overweight and obese horses have also been shown to be hyperleptinaemic and probably leptin-resistant (Buff *et al.*, 2002; Frank *et al.*, 2006; Carter *et al.*, 2009c; Kearns *et al.*, 2006). Similarly to people, blood leptin



concentration is proportional to adiposity in horses and has been shown to increase non-linearly with body condition score, in agreement with our findings of a non-linear increase in body fat with BCS (Kearns *et al.*, 2006; Huff *et al.*, 2009; Janssens and Van Weyenberg, 2010; Dugdale *et al.*, In Press b). However, not all obese horses are hyperleptinaemic/leptin resistant, just as not all obese horses are insulin resistant (Johnson *et al.*, 2004; Huff *et al.*, 2009). On this basis, caution should be applied if plasma leptin concentrations are to be considered as proxy measures of body fat contents.

Furthermore, total insulin secretion (basal + phasic meal-stimulated) has been shown to be directly proportional to body fat content in humans and horses, although, as noted above, not all obese individuals are insulin resistant (Frank *et al.*, 2006; Carter *et al.*, 2009c; Woods and D'Alessio, 2008). High insulin concentrations can cause insulin resistance by mechanisms including desensitisation of and/or internalisation of insulin receptors (Kolterman *et al.*, 1979; Rizza *et al.*, 1985), but insulin resistance itself results in increased insulin production and/or reduced insulin clearance such that a vicious cycle is entered and the cause and effect relationships become blurred (Erdmann *et al.*, 2009; Toth *et al.*, 2010). Insulin can also stimulate leptin production, possibly indirectly through its trophic effects on WAT (Kolaczynski *et al.*, 1996; Bluher *et al.*, 2002), and leptin can impair insulin signalling (Muller *et al.*, 1997), such that a complex interaction occurs between these two adiposity signals.

Overall, however, the usual ability of both these adiposity signals (leptin and insulin) to increase the sensitivity of the central nervous system to satiety signals

seems to become impaired in obese states. In addition, other features of the neuro-hormonal control of appetite appear to become impaired in the obese state (English *et al.*, 2002; Dugdale *et al.*, In Press a; section 7.1). The treatment of obesity, besides dietary restriction and increased exercise, therefore often includes treatments aimed at ameliorating insulin resistance (type 2 diabetes mellitus) and, occasionally, leptin resistance (Roth *et al.*, 2008) or other endocrine targets (see below). For horses and ponies, laminitis may co-exist with obesity and insulin resistance and also requires consideration when developing a treatment plan (McGowan, 2008; Geor, 2010).

Until recently (Van Weyenberg *et al.*, 2008; Dugdale *et al.*, 2010), there was no experimental evidence regarding the safety and efficacy of dietary protocols for overweight and obese horses and ponies. Insufficient information was presented in the earlier study (Van Weyenberg *et al.*, 2008) however, to enable translation into practice. The later study (Dugdale *et al.*, 2010) was devised to be easily applicable in a practical situation and concluded that dietary restriction to 1% of outset body mass (BM) as daily dry matter intake (DMI) produced a slow but steady weight loss at an overall rate of around 1% of actual outset body mass per week over the 12 weeks. Similar rates of weight loss have been targeted for people and small animals (German *et al.*, 2007). About half of the lost body mass was comprised of fat (assessed by deuterium oxide dilution). All animals remained healthy throughout the trial despite initial concerns over the possible induction of stress-induced gastric ulcers and stereotypic behaviours. Environmental enrichment strategies to help prolong meal times and reduce boredom are commonly suggested to offset these problems, including the use of multiple-layered haynets and providing several meals a day rather than two, where possible (Geor, 2010). In a follow-on study by our group (Curtis *et*

*al.*, 2010), overweight and obese mature horses and ponies, of various breeds and sexes, were studied over 16 weeks of dietary restriction to 1.25% actual BM as daily DMI. At this less severe dietary restriction, some animals appeared resistant to weight loss, which was not associated with breed or sex. However, in both studies, even after minimal weight (fat) loss, there was some improvement in insulin sensitivity as assessed by basal insulin and glucose concentrations (Dugdale *et al.*, 2010) and by the CGIT (results not shown).

In practice, weight loss is usually encouraged by a combination of dietary (energy) restriction and exercise, although exercise may not always be possible for horses and ponies suffering from laminitis. Exercise, in addition to increasing energy expenditure, also improves insulin sensitivity (Schmidt and Hickey, 2009). Consideration of dietary restriction, however, necessitates careful thought and, ideally, appreciation of the energy expenditure of the animal concerned which, at least for non-obese ponies, may vary somewhat with season (Dugdale *et al.*, In Press a), and evaluation of the nutrient and gross energy contents and the glycaemic/insulinaemic index and even glycaemic load of the food to be offered (Geor, 2010). When scaled for actual body mass, obese individuals usually have lower basal energy requirements than their leaner counterparts, with obese Native pony mares having only half the maintenance energy requirements of non-obese ponies (Bines *et al.*, 1969; NRC, 1990; Dugdale *et al.*, In Press a).

Foods of high fibre and low energy density and of low glycaemic/insulinaemic index are usually recommended for dietary restriction in *Equidae*, therefore forage-based rather than concentrate/cereal-based rations are preferred (Hoffman *et al.*, 2003;

Geor, 2010). Conserved forage can be soaked to reduce its energy density, but the leaching of soluble protein and carbohydrates depends upon fibre length, volume of water used, agitation during soaking and total time in soak and cannot easily be predicted (Longland *et al.*, 2009). In addition, minerals and vitamins can be lost, such that balancers are recommended (NRC, 2007). An alternative strategy to dilute the energy density of a ration is by the addition of low-energy forages such as straw, although there have been some concerns about gastric ulcers (Durham, 2010) and intestinal impaction colics by these means, especially when sudden management changes are imposed, including the provision of straw bedding (Mair and Hillyer, 1997; Goodwin *et al.*, 2002). Interestingly, the caloric dilution of rations with relatively indigestible sawdust has previously been reported (Laut *et al.*, 1985), and voluntary intake of wood shavings, provided as bedding for feed-restricted animals, was observed in one of this group's studies, seemingly without adverse effects (Curtis *et al.*, In Press).

In formulating energy-restricted rations to promote weight loss in *Equidae*, care must be taken not to limit dietary protein content because lysine, a conditionally-essential nutrient, may become limiting and restrict the production of L-carnitine which then reduces the animal's ability to metabolise fatty acids (especially medium and long-chain), through beta oxidation. Deficient protein provision may also result in excessive lean tissue loss, especially where physical exercise is not possible (Dugdale *et al.*, 2010). Protein, vitamin and mineral balancers are therefore commonly provided where dietary intake is reduced by dietary dilution (Curtis *et al.*, 2010; Geor, 2010). Furthermore, dietary protein itself may promote satiety and some amino acids improve insulin sensitivity (Storlien *et al.*, 2000; Potier *et al.*, 2009). High protein

diets may also enhance weight loss in people and small animals, but may be less useful for *Equidae*, because, as herbivores, horses and ponies maintain a certain requirement for dietary fibre, part of which may be to fulfil a requirement for chewing to wear down their continuously erupting teeth (NRC, 2007; Ellis, 2010; German *et al.*, 2010).

Although reduced energy intake is required to promote weight loss, there are some concerns over the long-term ability to maintain weight loss. In the medium term, reduced energy intake can reduce basal energy expenditure (i.e. basal metabolism), which would require further dietary energy restriction to maintain weight loss and so on, perhaps explaining the plateau of weight loss during dieting and weight rebound after dieting which are familiar to so many human dieters (Ramsey and Hagopian, 2006; Van Weyenberg *et al.*, 2008). Whether this decrease in basal energy expenditure is sustained for long periods after termination of dietary restriction remains to be determined. Whatever the result, it seems that dietary restriction alone is often insufficient in achieving and maintaining a leaner target body weight. Long-term dietary energy restriction, however, has been shown to promote longevity in several species (rodents, fish, flies, yeast), through mechanisms which may include reduced inflamm-aging (possibly secondary to reduced basal metabolic rate and reduced mitochondrial free radical production), improved insulin sensitivity, and alterations in neuroendocrine function (Weindruch *et al.*, 1986; Heilbronn and Ravussin 2003; Ingram *et al.*, 2006; Civitarese *et al.*, 2007). Biomarkers of inflamm-aging have been shown to be relatively increased in obese individuals, including horses, suggesting that those of leaner body composition should have reduced

diseases associated with oxidative damage and greater life expectancy (Adams *et al.*, 2009).

Further strategies to promote weight loss in horses and ponies have been reviewed (Geor, 2010), but where limited access to grazing is allowed, if this is without a grazing muzzle, care must be taken to guard against compensatory excessive forage intake because ponies were found to eat larger meals and more quickly when food was offered over a 3 month period of dietary restriction (Dugdale *et al.*, 2010). Owners must also be educated to reduce/remove the provision of ‘treats’ such as apples and carrots and to stop providing unnecessary thermal insulation in the form of rugs, which reduce energy expenditure. In fact, if they can be persuaded to trace-clip their animal in winter, this also helps promote energy expenditure to aid weight loss. Owners should be encouraged to monitor changes in their animals and if body weight itself is not directly measurable, then body girths provide a useful surrogate, whether translated into body weight measures (by use of weight tapes), or not. If animals prove to be ‘resistant’ to weight loss, further dietary restriction can be implemented (Curtis *et al.*, 2010).

Where horses and ponies fail to respond to dietary restriction, especially in cases where exercise is contra-indicated and animals are shown to be insulin resistant, then additional strategies will be required. These may include treatments to increase basal energy expenditure such as thyroid hormone (levo-thyroxine), or those to address the insulin resistance, such as metformin (a biguanide compound used in the treatment of type 2 diabetes mellitus in man) (Flier *et al.*, 2000; Frank *et al.*, 2005 & 2008; Durham *et al.*, 2008). There are interactions between leptin and other endocrine

(corticotrophic, gonadotrophic, somatotrophic and thyrotrophic) axes; and some unique aspects of the interaction between the thyrotrophic axis and leptin in horses have been discussed in terms of conferring an evolutionary advantage during seasonal availability of food (Flier *et al.*, 2000; Cartmill *et al.*, 2003; Buff *et al.*, 2007a and b). However, the efficacy of thyroid hormone supplementation in insulin resistant obese *Equidae* for improving insulin sensitivity and hastening adipose tissue loss requires more thorough evaluation (Frank *et al.*, 2008).

The exact mechanism of action of the biguanides remains to be discerned, but metformin is often described as an insulin-sensitiser or insulin-mimetic (Matthaei *et al.*, 2000). Although improvement in insulin sensitivity has been reported in insulin resistant horses following treatment with metformin (Durham *et al.*, 2008), pharmacokinetic data have highlighted its poor oral bioavailability in the horse (Hustace *et al.*, 2009). Further work in horses is necessary, however, to determine whether this poor oral bioavailability is due to poor absorption or very rapid renal excretion; and may simply warrant careful consideration of doses and dosing intervals for metformin to be effective in this species (Hustace *et al.*, 2009). Other treatments for insulin resistance in humans have been critically reviewed by Matthaei and colleagues (2000). Although thiazolidinediones appear to have been successful in the treatment of insulin resistance in people, one of them, rosiglitazone, was recently withdrawn from the market over safety concerns due to cardiac failure (Matthaei *et al.*, 2000). Dietary supplements such as magnesium, chromium, L-carnitine and cinnamon have been recommended in insulin resistant (type 2 diabetic) people and have also been suggested for use in insulin resistant horses, although may only be of benefit where there is pre-existing deficiency (Champagne, 2008; Geor, 2010;

Chameroy *et al.*, In Press). Potential herbal treatments have also recently been reviewed (Tinworth *et al.*, 2010).

In the war against human obesity, a wide range of pharmaceutical interventions have been, and continue to be, explored even though many of the products have been removed from the market due to the risks (usually in the form of adverse cardiovascular effects), outweighing the potential benefits (Cooke and Bloom, 2006). The drug classes investigated have included: those to suppress appetite (monoaminergic agonists or mimetics [amphetamine-based compounds and sibutramine were withdrawn] and cannabinoid receptor antagonists [rimonabant was also withdrawn]); those to reduce nutrient digestion and absorption (guar gum and pancreatic lipase inhibitors [orlistat remains available as an over-the-counter medication but has a revised safety data sheet]); and those to increase basal metabolic rate (amphetamines [withdrawn], thyroxine [prohibited for treatment of euthyroid people], dinitrophenol [uncouples oxidative phosphorylation in mitochondria; withdrawn]).

Amylin analogues (e.g. pramlintide), may improve leptin sensitivity and may help in human diabetes (types 1 and 2) where amylin can be deficient. Amylin is co-released with insulin from pancreatic beta cells and is regarded as an adiposity signal but may also have a role as a satiety signal. Recently there has been growing interest in liraglutide, a new analogue of glucagon-like peptide-1 (GLP-1, which is one of the incretins), which increases satiety (although at higher doses causes nausea and vomiting), and has been used for the treatment of human type 2 diabetes and also to



improve weight loss (Bray, 2009). However, many other avenues are being explored (Cooke and Bloom, 2006).

Novel therapies using ribonucleic acid interference (RNA<sub>i</sub>) to effectively silence gene expression, provide exciting new strategies for body fat control (Leonardsson *et al.*, 2004; Nofsinger *et al.*, 2008). Adipocyte apoptosis and subsequent infiltration of WAT with macrophages, themselves the source of many of the pro-inflammatory adipokines present in obese and insulin resistant states, may also become a target for therapeutic genetic intervention (Heilbronn *et al.*, 2004; Alkhouri *et al.*, 2010).

One area of interest to this author is how brown fat and thermogenesis (stimulated by beta3 agonists) fits into the picture (Fleury *et al.*, 1997; Grujic *et al.*, 1997; Fruhbeck *et al.*, 2009). Brown adipose tissue (BAT) is highly vascularised and innervated (sympathetic nervous system); wherein the brown adipocytes contain large numbers of mitochondria (mainly responsible for the brown appearance of the tissue) that contain an important uncoupling protein (UCP-1) which allows diversion of the metabolic pathways from the production of ATP (a cellular energy store) to the production of heat, enabling an important function of BAT in non-shivering thermogenesis (Pond, 1998). Although BAT diminishes with age, it may have roles in appetite regulation, the control of body weight/development of obesity and also in fever (Trayhurn *et al.*, 1982; Seydoux, 1983; Himms-Hagen, 1995; Cannon *et al.*, 1998; Himms-Hagen, 2001). While present in human neonates, lambs, kids and calves, functional brown adipose tissue has not been demonstrated in equine, porcine, canine or feline neonates (Ousey *et al.*, 1992; McDonald *et al.*, 2002). Whether BAT

and WAT can inter-differentiate remains an intriguing possibility for anti-obesity therapies (Danforth and Himms-Hagen, 1997; Pond, 1998).

Although the plethora of adipokines may seem likely targets for therapeutic intervention, we have yet to discover more about their multiple effects, both paracrine and endocrine, and their interactions (Schwartz *et al.*, 2000; Goralski *et al.*, 2007; Kralisch *et al.*, 2007; Lago *et al.*, 2009; Kos and Wilding, 2009; Bing *et al.*, 2010).

As a last resort, bariatric surgery (gastric banding or gastric reduction or bypass) is available for morbidly obese people where other treatments fail, although anaesthesia in such patients poses huge risks too (Adams and Murphy, 2000; Lotia and Bellamy, 2008). Gastric balloons, hydrogels or pseudobezoars are alternatives to radical surgery.

Bariatric surgery, associated with its own complications (Luber *et al.*, 2008), is not currently offered for the treatment of obesity in animals and would be improbable for *Equidae* due to lack of easy surgical access to the stomach, although two drugs have recently been licensed to aid weight loss in dogs: dirlotapide and mitratapide. These are inhibitors of enterocyte microsomal triglyceride transfer protein (MTP) which normally facilitates intestinal fat absorption and formation of chylomicrons for release into the lymphatics. Both enhance the weight loss achieved by dietary restriction, through reduced fat absorption and also by appetite reduction (through mechanisms possibly including peptide YY and GLP-1 release) (Gossellin *et al.*, 2007). Unlike pancreatic lipase inhibitors, steatorrhoea is not a side effect. The natural

herbivorous equine diet contains little fat, so similar strategies would be unlikely to be beneficial.

The multiple interactions between adipokines and the neuro-hormonal axes make it inevitable that one single treatment strategy will never be effective in all cases of obesity or insulin resistance. The old adage that ‘prevention is better than cure’ has never been more true! Future directions will, therefore, necessarily involve aspects of education for horse/pony owners, breeders and show judges, alongside veterinary surgeons. To this end, work with equine welfare charities and equine nutritionists at horse feed companies, with their high public profiles, will be paramount in helping to discern and disseminate new knowledge.

## **7.7 Conclusions**

The studies reported in this thesis have provided some insights into the basic adipobiology of Welsh mountain pony mares. For the first time, appetite was shown to be seasonally-modulated in non-obese, but not obese, ponies under domestic conditions: appetite for obese ponies averaged only ~2% of BM as DMI. Furthermore, we demonstrated that natural winter weight loss was negated by providing shelter and *ad libitum* access to food, thus proving one hypothesis for the development of obesity in Native animals kept under modern husbandry techniques. The first evidence of seasonal variation in fat-patterning also became apparent, perhaps suggesting some thermoregulatory adaptation because subcutaneous sites of fat deposition were favoured during winter, whereas internal sites were favoured during summer.

Dietary restriction to 1% BM as DMI over 12 weeks resulted in an overall rate of weight loss of 1% of outset BM per week, with no adverse effects on health but, despite an average loss of 30kg BM, no change in BCS was discerned. Several of the studies revealed that body fat was non-linearly related to BCS; validation of the D<sub>2</sub>O dilution technique for the measurement of total body water and body fat was instrumental in facilitating these investigations. The non-linear relationship between body fat and BCS, and the wider variation in body fat content at higher ( $\geq 7/9$ ; 'obese') scores, were responsible for the reduced sensitivity of body condition scoring to predict body fat content once animals were classed as obese. Observations made in these studies enabled development of a novel BCS system (E-FATS), which has the potential to overcome both the non-linearity of BCS systems and their problems associated with ambiguous descriptors.

Field testing of the novel E-FATS system, aided by the newly validated D<sub>2</sub>O dilution technique for measuring body fat, will enable any necessary modifications. If E-FATS can be shown to be a more accurate and precise predictor of body fat content (at least at stable body condition), than previous systems, it should facilitate future studies of changing body condition and body fat. In addition, once body fat can be easily measured, those percentages optimal for health and performance can begin to be investigated in order to improve equine welfare.

## **7.8 Future study interests**

My particular areas of future work at the University of Liverpool include: field-testing of the new BCS system; further data collection from attendees of the 'Fat

Attack' clinic; and investigation of the links between obesity and colic due to small intestinal strangulation by pedunculated lipomas.

### **To the future**

Prevention is better than cure, so they say  
And no doubt this is still true today.  
But both cause and effect of obesity  
Require much more attentive study.

To this end, I would state,  
That matters of late  
Require my attention elsewhere.  
But to help, where I can  
Would be part of my plan,  
In those parts of my time they call 'spare'.

To focus on colic  
Would be part of my frolic,  
With welfare concerns, paramount.  
And to pass on the message  
That fat is no blessing  
When present in excess amount.

Respiratory function requires a mention  
When the lungs get squashed up by much fat.  
How V and how Q fare, would warrant attention  
In the war against excessive WAT.

No one magic bullet or true panacea  
Will likely be found as a cure  
For all of these ills, with which fat can kill,  
Needing several approaches for sure.

With this end in sight,  
We must fight the good fight  
And continue as best as we can,  
To devote our resources  
To studies of horses  
And fulfil some of the goals of our plan.

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# General Appendix

## **KoneLab 20i Biochemistry analytical methods**

### **Glucose**

Glucose was determined by the hexokinase method. That is, glucose is phosphorylated to glucose-6-phosphate in a reaction catalysed by hexokinase and ATP. The glucose-6-phosphate so formed is oxidised to 6-phosphogluconate by glucose-6-phosphate dehydrogenase in the presence of NAD, an equimolar amount of which is reduced to NADH. Reduction of NAD (to NADH) is accompanied by an increase in absorbance at 340nm, allowing quantification of the amount of glucose present.

**Measuring range:** 0.3-40.0mmol/L; extended by sample dilution.

**Detection limit:** 0.1mmol/L

### **Cholesterol**

Cholesterol esters are enzymatically hydrolysed to cholesterol and free fatty acids by cholesterol esterase. Free cholesterol, including that already present in the sample, is then oxidised to cholest-4-en-3-one and hydrogen peroxide by cholesterol oxidase. The hydrogen peroxide reacts with hydroxybenzoic acid and 4-aminoantipyrine (under the influence of peroxidase), to form a chromophore (quinoneimine dye), which can be measured at 500-550nm.

**Measuring range:** 0.2-15.0mmol/L

**Detection limit:** 0.1mmol/L

### **Triglycerides**

Lipoprotein lipase hydrolyses triglycerides to free fatty acids and glycerol. Glycerol is then phosphorylated to glycerol-3-phosphate (by glycerokinase), which is then oxidised to dihydroxyacetone phosphate and hydrogen peroxide (by glycerol-3-phosphate oxidase). The hydrogen peroxide reacts with 4-chlorophenol and 4-aminoantipyrine (under the influence of peroxidase), to form a quinoneimine dye, which can be quantified at 510nm.

**Measuring range:** 0.05-11.00mmol/L

**Detection limit:** 0.02mmol/L

### **Urea**

Urease catalyses the hydrolysis of urea in the presence of water to produce ammonia and carbon dioxide. Glutamate dehydrogenase then catalyses the reaction between ammonia and alpha-ketoglutarate, in the presence of NADH, to produce L-glutamate. NADH is reduced to NAD in proportion to the urea concentration in the sample. The decrease in NADH is accompanied by a decrease in absorbance at 340nm, which can be measured.

**Measuring range:** 3.0-25.0mmol/L

**Detection limit:** 1.1mmol/L

### **Creatinine**

Measurement is based on the Jaffe method whereby creatinine forms a red colour with an alkaline picrate solution. The method measures the rate of formation of the coloured complex, measuring absorbance at 510nm.

**Measuring range:** 10-800umol/L

**Detection limit:** 5umol/L

### **Total protein**

Plasma samples give results about 1-5g/L higher than serum samples due to fibrinogen content. Plasma samples were used in the studies reported in this thesis.

Protein forms a coloured complex with cupric ions in alkaline solutions. The formation of the complex is measured at 540nm. The method utilises EDTA as a chelating agent and stabilising agent for cupric ions.

**Measuring range:** 3-100g/L (=0.3-10g/dL)

**Detection limit:** 1g/L (=0.1g/dL)

### **Albumin**

When albumin reacts with a coloured dye, bromocresol green (BCG), a coloured product is formed whose colour intensity is measured by absorbance at 600nm.

**Measuring range:** 2-45g/L (=0.2-4.5g/dL)

**Detection limit:** 1g/L (=0.1g/dL)

### **Bile acids**

The enzyme 3- $\alpha$ -hydroxysteroid dehydrogenase converts bile acids, in the presence of Thio-NAD, to 3-keto steroids and Thio-NADH. (The reaction is reversible so that 3- $\alpha$ -HSD can convert 3-keto steroids and Thio-NADH into bile acids and Thio-NAD). In the presence of excess NADH, the enzyme cycling occurs efficiently and the rate of formation of Thio-NADH is therefore dependent upon the bile acid concentration and is determined by measuring the specific change of absorbance at 405nm.

**Measuring range:** the method is linear up to a concentration of 150umol/L

**Detection limit:** 1umol/L

### **β-OH butyrate**

Serum samples (rather than plasma), must be used. In the presence of β-hydroxybutyrate dehydrogenase, β-OH butyrate reacts with NAD to form acetoacetate and NADH. The rate of formation of NADH is proportional to the concentration of β-OH butyrate and is measured at 340nm.

**Detection limit:** 0.1mmol/L

Analyses were done at VLA, Shrewsbury; all their QC/QA arrangements were therefore adhered to although they had no reference range for horses.

### **Non-esterified fatty acids (NEFA)**

Serum samples must be used. Acyl-coA synthetase catalyses the conversion of NEFA to fatty acyl-CoA thiol esters, in the presence of ATP, Mg and CoA. The acyl-CoA derivatives are oxidised by acyl-CoA oxidase to produce hydrogen peroxide and 2,3-trans-enoyl-CoA. Hydrogen peroxide, in the presence of peroxidase, results in the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline with 4-aminoantipyrine which produces a blue-purple product (quinoneimine) that absorbs light at 550nm. The NEFA concentration can therefore be determined by measuring the optical density (absorbance) at 540-550nm.

**Measuring range:** 0.01 - 4mmol/L; extended by sample dilution

**Detection limit:** 0.01mmol/L

### **Fructosamine**

This colorimetric assay is based on the ability of ketoamines to reduce nitrotetrazolium blue (NBT) to formazan in an alkaline solution. The rate of formation of formazan is directly proportional to the concentration of fructosamine. Uricase serves to eliminate uric acid interference and detergent eliminates matrix effects. The rate of the reaction is measured photometrically at 546nm.

**Measuring range:** 10-1000umol/L

**Detection limit:** 10umol/L

### **Neutral detergent fibre determination**

Neutral detergent fibre comprises the insoluble fibrous residue (hemicelluloses, cellulose, ligno-cellulose and lignin) after extraction/dissolution of soluble and easily digested components (soluble fibres [e.g. pectins], sugars and starches). The detergent solubilises proteins, EDTA chelates calcium and helps remove pectins during boiling and alpha amylase enables digestion of starch to soluble sugars.

#### ***NDF solution***

Disodium EDTA (ethylene diamine tetra-acetate dihydrate) (93.0g) and sodium borate decahydrate (34.0g) are dissolved in 3 litres deionised water in a 5L beaker during heating and stirring. Sodium dodecyl sulphate (SDS) (150.0g) are added and heating and stirring continued until this has dissolved. The beaker is then removed from the heat and 2-ethoxy ethanol (50ml) is added. Anhydrous di-sodium hydrogen phosphate (22.8g) is dissolved in 1 litre deionised water with heating and stirring. Once dissolved, this is added to the first solution and the final volume is diluted to 5 litres with deionised water. The solution is mixed well and the pH is checked: should be between 6.9 and 7.1. Adjustment should not be necessary.

#### ***Technique for samples with expected <5% fat***

Immediately prior to use, the requisite number of sintered glass crucibles (50ml capacity; porosity number 1), are placed into a muffle furnace and heated to 500°C for a minimum of 30min before being transferred to a desiccator to be allowed to cool. The crucibles are then weighed accurately (W1).

Between 0.50 and 0.55g of the well-mixed ground sample (ground to pass a 1mm screen) are weighed into a conical flask and 100ml of NDF solution are added. The flask contents are gently worked so as to break up any lumps and ensure all the dry sample is immersed in the solution. Alpha amylase enzyme (0.10ml) is then added to each flask. Prepared flasks are heated to boiling on a heating mantle; 'cold finger' condensers are inserted into the flask necks and the flasks are maintained under reflux for 60min, timed from the commencement of boiling.

Once the flask is removed from the hotplate, the contents are filtered, as soon as possible, through a pre-weighed sintered glass crucible using gentle suction (Buchner flask). The entirety of the flask contents must be transferred to the crucible, using hot water from a wash bottle and, if necessary, a rubber-tipped glass rod to remove particle adhering to the sides of the flask. The contents of the crucible are then washed twice with approximately 50ml boiling water.

The crucible is seated into a rubber cone (to prevent drainage through the sintered base) and 30ml hot (~80°C) water are added, followed by 0.10ml alpha amylase. The crucible is allowed to stand for 15min after which it is replaced in the filtration apparatus and the contents washed twice with ~50ml hot water and then twice with ~50ml acetone.

Finally, the crucible is placed into an oven (100°C) for a minimum of 5hr (or overnight). After drying, the crucible is transferred to a desiccator and allowed to cool before being weighed accurately (W2).

The crucible is then placed into a muffle furnace (500°C) for a minimum of 3hr, before being transferred to a desiccator, allowed to cool and accurately weighed again (W3).

Samples are analysed in duplicate. For internal quality control, standards are added to each run, for example digestive biscuits.

### *Calculations*

$$\% \text{ NDF} = \frac{(W2-W1)}{\text{Sample mass}} \times 100 \quad (1)$$

$$\% \text{ NDF (ash-free)} = \frac{W2-W3}{\text{Sample mass}} \times 100 \quad (2)$$

The second calculation (equation 2) was used to determine the NDF (ash-free) content of food and faecal samples for all apparent digestibility studies reported in this thesis.

# Published Papers

Assessment of body fat in the pony: Part I. Relationships between the anatomical distribution of adipose tissue, body composition and body condition.

Dugdale AH, Curtis GC, Harris PA, Argo CM.

Equine Vet J. 2011 Mar 4. doi: 10.1111/j.2042-3306.2010.00330.x. [Epub ahead of print]  
PMID: 21496091

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Assessment of body fat in the pony: Part II. Validation of the deuterium oxide dilution technique for the measurement of body fat.

Dugdale AH, Curtis GC, Milne E, Harris PA, Argo CM.

Equine Vet J. 2011 Mar 4. doi: 10.1111/j.2042-3306.2010.00327.x. [Epub ahead of print]  
PMID: 21496088

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Effect of dietary restriction on body condition, composition and welfare of overweight and obese pony mares.

Dugdale AH, Curtis GC, Cripps P, Harris PA, Argo CM.

Equine Vet J. 2010 Oct;42(7):600-10. doi: 10.1111/j.2042-3306.2010.00110.x. Erratum in:  
Equine Vet J. 2011 Jan;43(1):121.

PMID: 20840575

[PubMed - indexed for MEDLINE]

Effects of season and body condition on appetite, body mass and body composition in ad libitum fed pony mares.

Dugdale AH, Curtis GC, Cripps PJ, Harris PA, Argo CM.

Vet J. 2010 Dec 10. [Epub ahead of print]